## REMARKS

In the Office Action dated July 17, 2007, Claims 1-25 are pending and under examination. Claim 1 is objected to for reciting the acronym "APC". Claims 1-8, 10 and 12-16 are rejected under 35 U.S.C. §103(a) as allegedly obvious over Bochan et al. (*Transplant Proc* 31:690-91, 1999) in view of Burt et al. (*Autoimmunity Rev* 1:133-38, May 2002). Claim 11 is rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Bochan et al. in view of Burt et al., and further in view of U.S. Patent No. 6,428,782 to Slavin et al. Claims 1-25 are rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enabling support. Claims 1-9 and 18-25 are rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite. Claims 18-25 are rejected under 35 U.S.C. §101 for improper definition of a process. Claim 21 is objected to for allegedly lacking insufficient antecedent basis.

This Response addresses each of the Examiner's objections and rejections. Applicants therefore respectfully submit that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

Claim 1 is objected to for reciting an allegedly undefined acronym "APC". Applicants have amended Claim 1 to recite "antigen presenting cell (APC)". Support for the amendment is found in the specification, on page 3, line 8. No new matter is introduced by the amendment to Claim 1. As such, the objection to Claim 1 is obviated and withdrawal thereof is respectfully requested.

Claims 1-8, 10 and 12-16 are rejected under 35 U.S.C. §103(a) as allegedly obvious over Bochan et al. (*Transplant Proc* 31:690-91, 1999) in view of Burt et al. (*Autoimmunity Rev* 1:133-38, May 2002).

In the first instance, Applicants respectfully submit that the present invention is partly

predicated on the recognition that transplantation of syngeneic or autologous HSCs infected with proinsulin can induce immune tolerance. Transplantation of syngeneic or autologous HSCs avoids the risk of graft rejection and represents a safe strategy for treating or preventing autoimmune diseases including diabetes.

Applicants observe that Bochan et al. teach experiments in treating rats with streptozotocin (STZ)-induced diabetes, which involves collecting hematopoetic stem cells (HSCs) from rat bone marrow, infecting the HSCs with a recombinant AAV vector expressing rat proinsulin, and reintroducing the transfected HSCs to rats with STZ-induced diabetes. The Examiner acknowledges that the primary Bochan et al. do not teach autologous transplantation of HSCs.

However, the Examiner alleges that the secondary reference to Burt et al. merely mention autologous transplantation of HSCs. In this regard, Applicants observe that Burt et al. merely mention that autologous HSC tranplants, in contrast to allogenic HSC transplants, are relatively safe. See page 135, middle of left column of the Burt et al. reference. Applicants observe that the cited Burt et al. reference was published in 2002. Applicants respectfully submit that in 2002 autologous HSC transplantation was yet to be tested in the treatment of diabetes. Indeed, Burt et al. in a later publication (Burt et al, *Arthritis and Rheumatism* 50(8):2466-70, 2004 ("Burt et al. 2004") (copy enclosed as **Exhibit 1**) clearly indicated that the results of autologous HSC transplantation were unpredictable and varied according to the disease and the conditioning regimen used. See page 2466 of **Exhibit 1**. **Exhibit 1** in fact teaches that allogeneic transplantation resulted in marked amelioration of rheumatoid arthritis (RA) with no remission even after one year. In fact, it was well documented, particularly at the time the present application was filed, that despite the improved safety and remission of certain

autoimmune disease following autologous HSC transplantation, remission was not usually sustained and early relapses were common. See review articles by, e.g., Burt et al. (*Blood* 99(3):768-84, 2002) (copy enclosed as **Exhibit 2**) and Hough et al. (*British Journal of Haematology* 128:432-59, 2004) (copy as **Exhibit 3**). For example, prior to the present invention, autologous transplantation had been found to be relatively safe and well tolerated in RA, but a complete remission had been unusual and relapse commonly occurred within 1 to 2 years. Indeed, autologous transplantation in scleroderma studies had been found to result in high mortality rates. As such, the lack of sustained responses in many patients led some investigators to explore allogeneic HSC transplantation which resulted in a complete remission of RA at over 5 years follow-up (see **Exhibit 3,** last paragraph of page 443). Thus, prior to the present invention, more durable responses had been observed following allogeneic transplantation in autoimmune diseases. To this extent, Applicants respectfully submit that it was not common general knowledge at the time the present application was filed that autologous transplantation would be more effective and safer than allogeneic transplantation. Accordingly, a person skilled in the art would not have been motivated to combine Bochan et al. with Burt et al. Even assuming, *argendo*, that one skilled in the art would combined the two cited references, there would have been no expectation of success because the result of autologous HSC transplantation would have been unexpected, as indicated by Burt et al. (2004) (**Exhibit 1**).

Furthermore, similar to the Bochan et al. reference, the secondary reference to Burt et al. does not describe autologous, antigen(proinsulin)-specific HSC transplantation.

Accordingly, Applicants respectfully submit that contrary to the Examiner's allegation, the secondary reference to Burt et al. does not ameliorate the deficiencies of the primary reference to Bochan et al.

Therefore, the rejection of Claims 1-8, 10 and 12-16 under 35 U.S.C. §103(a) as allegedly obvious over Bochan et al. in view of Burt et al. is overcome and withdrawal thereof is respectfully requested.

Claim 11 is rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Bochan et al. in view of Burt et al. as applied to Claims 1-8, 10, 12-16 above, and further in view of U.S. Patent No. 6,428,782 to Slavin et al. The Examiner alleges that Slavin et al. teach that it was routine in the art to use cytokine-mediated mobilization to increase the numbers of HSCs extracted from bone marrow.

Applicants respectfully submit that this rejection cannot stand because Claims 1-8, 10 and 12-16 are not obvious over Bochan et al. in view of Burt et al. as discussed above. Therefore, the rejection of Claim 11 under 35 U.S.C. §103(a) as allegedly unpatentable over Bochan et al. in view of Burt et al., and further in view of Slavin et al. is overcome and withdrawal thereof is respectfully requested.

Claims 1-25 are rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the enablement requirement. The Examiner alleges that the specification fails to teach that it is the proinsulin-induced immune response that asserted a therapeutic effect in a subject having insulin-dependent diabetes.

In this regard, Applicants respectfully direct the Examiner's attention to the specification, e.g., in Example 2 on page 22, which clearly demonstrates that transplantation of autologous bone marrow cells (containing HSCs) transfected with proinsulin prevented diabetes. See also Example 4 on page 23 of the specification.

In addition, relying on Steptoe et al. (*J. Immunol* 168: 5032-41, 2002), the Examiner alleges that administering dendritic cells expressing proinsulin would likely to induce or

exacerbate autoimmune diabetes rather than treating it. The Examiner alleges that the claimed methods are not enabled in view of the contradictory evidence found in the art.

In the first instance, Applicants observe that although diabetes-prone nonobese diabetic (NOD) mice were reported to have increased dendritic cells (DC), which contribute to autoimmune disease susecptibilty, those dendritic cells did not express proinsulin. Importantly, the present invention employs APCs (including DC) expressing an autoantigen, such as proinsulin, to induce immune tolerance and thereby treating or preventing autoimmune diseases. Therefore, Applicants respectfully submit that there is no contradiction between the present invention and the teaching of Steptoe et al.

In addition, Applicants submit that a co-author of the Steptoe et al. reference, Dr. Ray Steptoe, is also a named co-inventor in the present application. Applicants observe that Steptoe et al. teach that there was a defect in autoimmune diabetes-prone NOD mice, wherein increased proportions of certain DC were found compared with recombinant congenic non-obese diabetes-resistant mice. However, Steptoe et al. do not describe the induction of immune tolerance. As mentioned above, the DC described in Steptoe et al. does not express an autoantigen, such as proinsulin, to induce immune tolerance and thereby treat or prevent autoimmune disease. Indeed, Dr. Ray Steptoe in another publication showed that DC alone were not sufficient without the presence of proinsulin to suppress diabetes. See Steptoe et al, *Diabetes* 54:434-42, 2005. (copy enclosed as **Exhibit 4**). Therefore, Applicants respectfully submit that the specification provides sufficient guidance for one skilled in the art to make HSCs expressing an autoantigen and used them in the claimed method for preventing or treating insulin-dependent diabetes in a subject, without undue experimentation.

The Examiner states that Claims 9, 17 and 25 recite a humanized proinsulin derived

from non-human animals. However, the Examiner alleges that the specification fails to teach the structure of a "humanized" proinsulin or how to make a humanized proinsulin. The Examiner alleges that the term "humanized proinsulin", as recited in Claims 9, 17 and 25, lacks enabling support. In an effort to favorably advance prosecution, Applicants have canceled Claims 9, 17 and 25. As such, the enablement rejection with respect to Claims 9, 17 and 25 is moot.

In view of the forgoing, the rejection of Claims 1-25 under 35 U.S.C. §112, first paragraph, as allegedly lacking enabling support is overcome and withdrawal thereof is respectfully requested.

Claims 1-9, 18-25 are rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite. The Examiner alleges that Claims 1-9 are vague and indefinite because Claim 1 requires introducing into a subject in need a genetically modified APC, whereas the method step comprising administering HSCs or HPCs. It is unclear how introducing HSCs is associated with introducing APCs. The Examiner also alleges that Claims 1-9 are vague and indefinite because a step is missing in Claim 1, i.e., introducing the genetically modified HSCs back to the subject. The Examiner further alleges that Claims 18-25 do not set forth any steps involved in the method/process.

Applicants have amended Claim 1 and deleted Claims 18-25. Claim 1, as amended, clarifies that the recited HSCs and HPCs expressing pro-insulin develop into APCs. Claim 1, as amended, also recites a step of introducing the genetically modified HSCs/HPCs back to the subject. Support for the amendments is found in the specification on page 3, lines 8-17 and bridging paragraph of pages 304. No new matter is introduced by the amendment to Claim 1. Applicants respectfully submit that Claim 1, as amended, and Claims 2-9, which depend from Claim 1, are clear and not indefinite.

As such, the rejection of Claims 1-9 under 35 U.S.C. §112, second paragraph, is overcome. The rejection with respect to Claims 18-25 is moot in view of the cancellation of Claims 18-25. Withdrawal of the rejection under 35 U.S.C. §112, second paragraph, is respectfully requested.

Claims 18-25 are rejected under 35 U.S.C. §101 for improper definition of a process. The rejection is moot in view of the cancellation of Claims 18-25.

Claim 21 is objected to for allegedly lacking antecedent basis by reciting the limitation "said HSCs and/or HPCs." The objection is moot in view of the cancellation of Claim 21.

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

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# Induction of Remission of Severe and Refractory Rheumatoid Arthritis by Allogeneic Mixed Chimerism

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**This report describes the first allogeneic hematopoietic stem cell transplantation (HSCT) performed for the indication of rheumatoid arthritis (RA). We used nonmyeloablative allogeneic HSCT to treat a 52-year-old woman who had treatment-refractory RA and a poor prognosis (24 swollen and 38 involved joints). She was treated with fludarabine, cyclophosphamide, CAMPATH-1H, and CD34-selected HSCT (8 million CD34+ donor cells/kg); the donor was the patient's HLA-matched, rheumatoid factor–negative sister. One year post-HSCT, the patient has had no infection except dermatomal varicella-zoster virus infection and no acute or chronic graft-versus-host disease (GVHD). Her RA has remained in remission with no immunosuppressive or immunomodulatory medications. The patient is a mixed chimera, with 55% donor T (CD3+) cells and 70% donor myeloid (CD33+) cells. This is the first published report of allogeneic HSCT performed for the indication of RA. Mixed chimerism has resulted in marked amelioration of RA, without GVHD.**

Hematopoietic stem cell transplantation (HSCT) for the treatment of autoimmune diseases has been overwhelmingly autologous, due to safety reasons. Four hundred ninety-five autologous HSCTs, but no allogeneic HSCTs, for the treatment of autoimmune disease have been reported to the European Bone Marrow Transplant international registry database in Basel, Switzerland (Passweg J: personal communication). Results of autologous HSCT have varied according to the disease and the conditioning regimen used. For patients with rheumatoid arthritis (RA), use of a regimen of cyclophosphamide (200 mg/kg) and antithymocyte globulin has resulted in marked clinical improvement, although the majority of patients experience a relapse within 1–2 years after autologous HSCT (1–3). The regimen has been well tolerated, with no mortality reported in >70 patients with RA who underwent HSCT (4).

Because the long-desired goal of curing RA remains elusive with the current autologous HSCT regimens, we initiated a nonmyeloablative allogeneic HSCT protocol for patients with severe and refractory RA. The protocol is designed to minimize both conditioning regimen–related toxicity and graft-versus-host disease (GVHD) by using nonmyeloablative stem cell transplantation (NST) and a CD34-selected (i.e., lymphocyte-depleted) HLA-matched sibling graft, respectively. The goal of the treatment is to determine the effect of mixed chimerism, in which donor and recipient hematopoiesis coexists in patients with RA.

Case reports of allogeneic HSCT performed for a hematologic indication such as aplastic anemia in patients with coexistent RA have described a drug-free and durable complete remission in the majority of patients who were available for long-term followup (5,6). In those cases in which relapse occurred after allogeneic HSCT, the donor's rheumatoid factor (RF) status either was not reported, or the donor was RF positive (7,8). Despite their potential for being curative, allogeneic HSCTs are traditionally associated with a high risk of mortality, attributable to both the conditioning regimen and GVHD. Because of these toxicities, conventional allogeneic HSCT has not been considered appropriate for patients with RA (9). However, newer, less intensive methods of allogeneic stem cell transplantation (so-called nonmyeloablative transplantation) have been introduced in order to reduce morbidity and mortality

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(10). We used strategies that minimize the risk of both conditioning regimen–related toxicity and GVHD to achieve mixed chimerism, and now present the first published report of allogeneic HSCT using an HLA-matched sibling donor, for the indication of RA.

## CASE REPORT

The patient was a 52-year-old woman in whom prior autologous HSCT using a conditioning regimen of cyclophosphamide (200 mg/kg) and equine antithymocyte globulin (90 mg/kg) had failed 4 years earlier. Thereafter, she underwent treatment with infliximab, methotrexate, leflunomide, prednisone, and oral and intravenous cyclophosphamide, which subsequently failed. The patient then underwent NST with 8 million CD34+ donor cells/kg obtained from her HLA-matched, RF-negative sister. The donor graft was mobilized with granulocyte colony-stimulating factor (G-CSF; 10 $\mu$g/kg/day) for 6 consecutive days. Stem cell apheresis was performed on days 4, 5, and 6 of treatment with G-CSF. The total pre- and postselection CD34+ hematopoietic stem cell counts were $11.3 \times 10^6$/kg and $8.0 \times 10^6$/kg, respectively, and the pre- and postselection CD3+ T cell counts were $10.3 \times 10^8$/kg and $4.5 \times 10^4$/kg, respectively.

The protocol was approved by the US Food and Drug Administration under the Investigational New Drug number 10175 and by the Institutional Review Board. Inclusion criteria were as follows: 1) an established clinical diagnosis of RA according to the American College of Rheumatology (ACR) revised criteria (11), 2) >12 swollen joints from active RA, 3) >20 involved joints (swelling, tenderness, deformity, pain on motion, or decreased motion) despite treatment with methotrexate and/or leflunomide and a tumor necrosis factor $\alpha$ inhibitor, and 4) availability of an RF-negative HLA-matched sibling donor.

The conditioning regimen used was fludarabine (125 mg/m$^2$), cyclophosphamide (150 mg/kg), and CAMPATH-1H (20 mg). Selection of CD34+ enriched cells from the apheresis product was performed using the Isolex cell separator system (Baxter, Chicago, IL), with the goal of obtaining >10 million CD34+ cells/kg. After HSCT, prophylaxis against infection consisted of aerosolized pentamidine (administered monthly), daily oral vorconazole, and daily oral ganciclovir. Antibiotic prophylaxis was discontinued when the CD4 T cell count increased to >100 cells/ml.

Prior to NST, the patient had 24 swollen and 38 involved joints. Engraftment occurred on day 11. The transplant was uncomplicated, and she has not received a platelet or red blood cell transfusion since day 14. At the time of this report, 1 year post-HSCT, the patient's RA remains in remission. Her only infection was dermatomal varicella-zoster virus infection, which occurred 10 months after allogeneic HSCT and was successfully treated with oral acyclovir. Oral cyclosporin A (CSA) and oral mycophenolate mofetil (CellCept; Hoffman-La Roche, Nutley, NJ) were started 4 days before stem cell infusion as prophylaxis against both rejection (host-versus-graft) and GVHD. CSA and CellCept were discontinued 30 days and 9 months, respectively, after HSCT. There has been no evidence of either acute or chronic GVHD. Hematopoietic donor engraftment has been followed by variable-number tandem repeats of the apoprotein B locus from flow-sorted CD33+ myeloid and CD3+ T cells. Donor CD33+ engraftment was

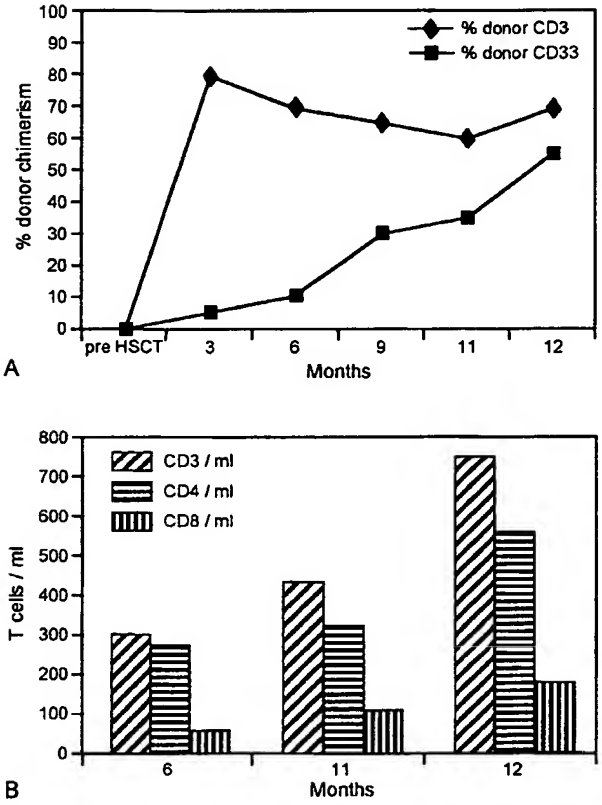


**Figure 1.** **A,** Mixed donor chimerism in T lymphocytes and granulocytes 3, 6, 9, 11, and 12 months after nonmyeloablative stem cell transplantation (NST). HSCT = hematopoietic stem cell transplantation. **B,** Total number of T cells in the patient's peripheral blood 6, 11, and 12 months after allogeneic NST.

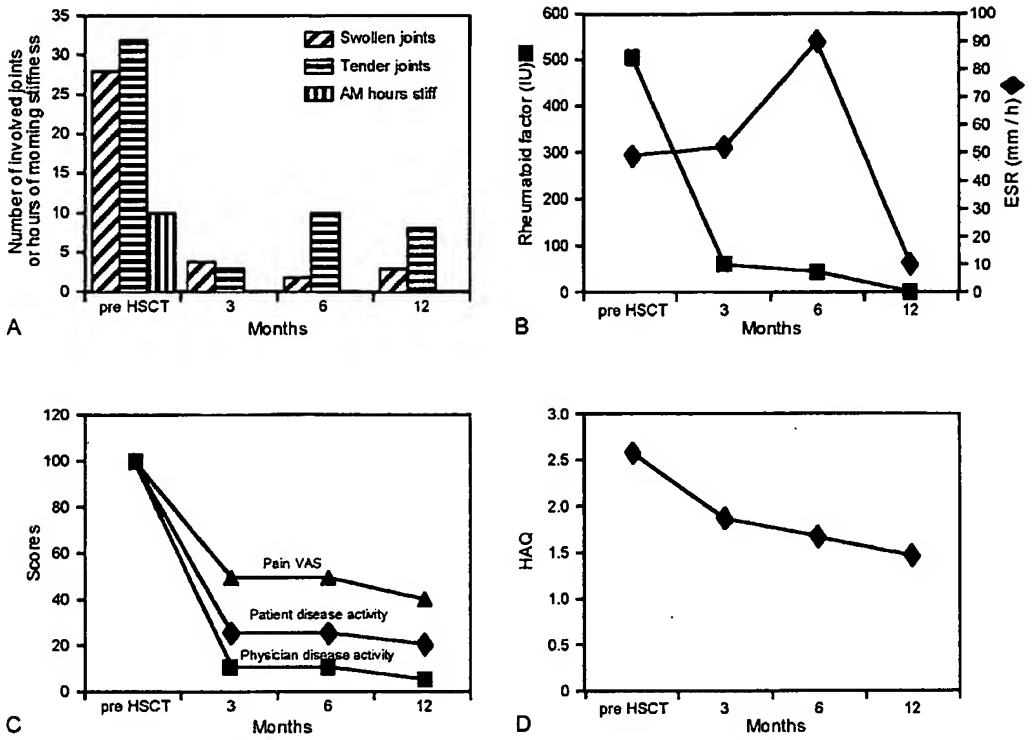


**Figure 2.** Clinical and laboratory course of the patient after nonmyeloablative stem cell transplantation (examined 3, 6, 9, and 12 months after transplantation). **A,** Number of swollen and tender joints, and duration of morning stiffness. **B,** Rheumatoid factor levels and erythrocyte sedimentation rate (ESR). **C,** Patient's and physician's global assessments of disease status, and patient's awareness of pain as measured on a visual analog scale (VAS). **D,** Health Assessment Questionnaire (HAQ) activities of daily living scores. HSCT = hematopoietic stem cell transplantation.

immediate and predominated early after HSCT, while donor CD3+ T cell engraftment gradually rose from 5% to 55% over 12 months after HSCT (Figure 1A). Immune reconstitution was monitored by the CD3+, CD4+, and CD8+ cell counts (Figure 1B). All antibiotics were discontinued 9 months after HSCT, when the CD4+ T cell count was >100/μl. One month later, dermatomal varicella-zoster virus infection developed but responded to treatment with oral acyclovir.

Morning stiffness, which had been present for approximately 10 hours daily prior to HSCT, completely disappeared before the patient was discharged from the hospital and has not recurred for more than 1 year after HSCT (Figure 2A). Before HSCT, the patient's swollen and tender joint counts were 28 and 32, respectively. The patient's swollen and tender joint counts markedly decreased prior to hospital discharge, and ACR 70% improvement (12) has been maintained for more than 12 months, in the absence of immunosuppressive therapy (Figures 2A and C). Golf ball–size rheumatoid nodules that were on the extensor surface of the patient's forearm pretransplantation disappeared gradually and were completely gone 9 months after HSCT (Figures 3A and B). The RF level and the erythrocyte sedimentation rate gradually normalized by 12 months after HSCT (Figure 2B). Scores for patient's assessment of pain on a visual analog scale (VAS), patient's assessment of disease activity on a VAS, and physician's global assessment of disease activity markedly improved beginning 3 months after HSCT (Figure 2C). The patient attributed her awareness of pain to permanent foot deformities due to prior erosive joint destruction that caused pain while walking. Before HSCT, the patient's score on the Health Assessment Questionnaire (HAQ) (13) was 2.6; by 12 months after HSCT, the score had improved to 1.5 (Figure 2D).

Skeletal radiographs obtained before HSCT and those obtained at the most recent evaluation (12 months after HSCT) did not differ. Radiographs of the right foot that were obtained before and after HSCT showed

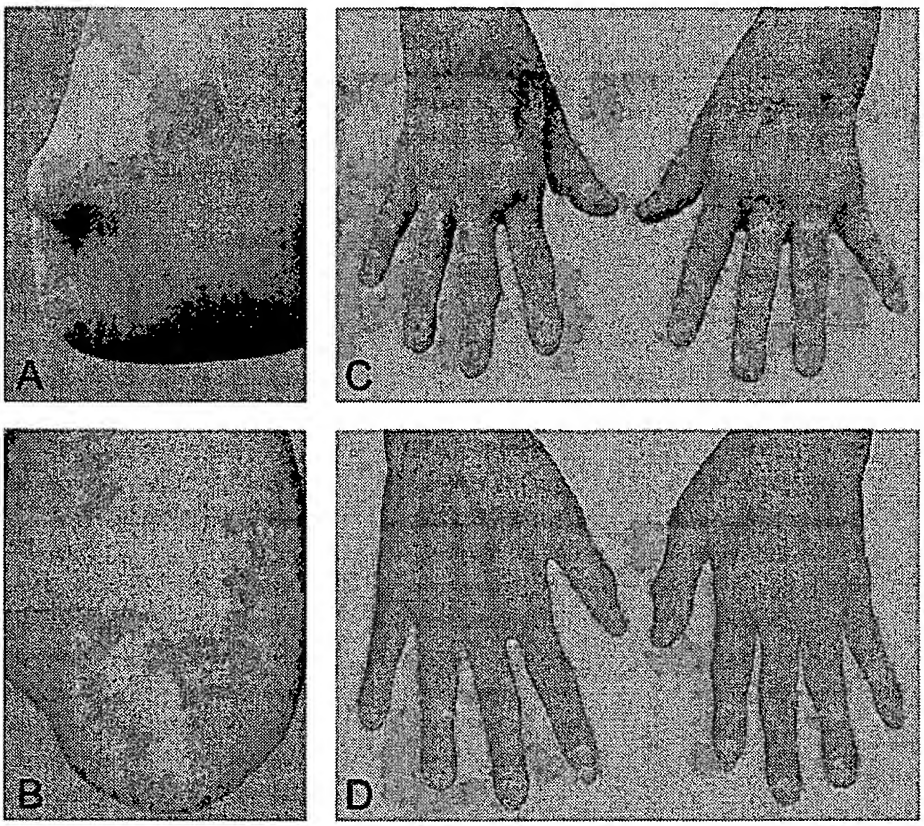


**Figure 3.** Photographs showing the patient's right elbow and hands before allogeneic stem cell transplantation (**A** and **C**) and 12 months after the procedure (**B** and **D**).

massive erosions of the first and fifth metatarsophalangeal joints, hammertoe deformity in digits 2–5 bilaterally, and severe hallux valgus with bunion formation. The left foot demonstrated dislocation of the second, third, and fourth metatarsophalangeal joints, hammertoe deformity of digits 2–5, and severe hallux valgus.

## DISCUSSION

Mixed chimerism (i.e., both recipient and donor hematopoiesis) induces remission of diabetes and lupus-like autoimmune diseases in animal models (14,15). Due to the older age of patients with RA, an NST regimen was designed to induce mixed chimerism with minimal conditioning regimen–related toxicity or risk of GVHD. To avoid transferring occult disease with the graft, the donor must be RF negative. The disappearance of rheumatoid nodules, RF, and morning stiffness, and normalization of the erythrocyte sedimentation rate are consistent with a complete remission. In our patient, the disappearance of RF correlated with increased donor T cell engraftment. Her tender and swollen joint counts improved by 70% according to the ACR criteria but did not completely normalize. Whether this is attributable to residual RA or to tenderness and swelling secondary to permanent deformities and cartilage/bone destruction that were present before HSCT is unknown. The VAS score for residual pain and the abnormalities on the HAQ are more likely secondary to irreversible joint damage and deformity, making some tasks such as walking on deformed feet painful and difficult. This conclusion is supported by the extensive and destructive joint changes that were observed on skeletal radiographs both before and after HSCT.

After 1 year of followup, these data suggest that NST using CD34-selected hematopoietic stem cells may be performed safely, without the development of GVHD or serious infection, and results in mixed chimerism with marked resolution of the disease manifestations of RA.

Further investigation of NST in patients with RA appears to be warranted.

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# Induction of tolerance in autoimmune diseases by hematopoietic stem cell transplantation: getting closer to a cure?

Richard K. Burt, Shimon Slavin, William H. Burns, and Alberto M. Marmont

**Hematopoietic stem cells (HSCs) are the earliest cells of the immune system, giving rise to B and T lymphocytes, monocytes, tissue macrophages, and dendritic cells. In animal models, adoptive transfer of HSCs, depending on circumstances, may cause, prevent, or cure autoimmune diseases. Clinical trials have reported early remission of otherwise refractory autoimmune disorders after either autologous or allogeneic hematopoietic stem cell transplantation (HSCT). By percentage of transplantations performed, autoimmune diseases are the most rapidly expanding indication for stem cell transplantation. Although numerous editorials or commentaries have been previously published, no prior review has focused on the immunology of transplantation tolerance or development of phase 3 autoimmune HSCT trials. Results from current trials suggest that mobilization of HSCs, conditioning regimen, eligibility and exclusion criteria, toxicity, outcome, source of stem cells, and posttransplantation follow-up need to be disease specific. HSCT-induced remission of an autoimmune disease allows for a prospective analysis of events involved in immune tolerance not available in cross-sectional studies. (Blood. 2002;99:768-784)**



## Autoimmunity: definition

Autoimmunity arises from the pathologic reaction of B-cell–derived antibodies and/or T cells to self-epitopes. Proof of an autoimmune pathogenesis requires adoptive transfer of disease by either immune cells or antibody.[1,2] Transplacental or iatrogenic transfer of autoreactive antibodies may cause disease. This condition was first shown in Harrington's self experimentation using plasma from a patient with idiopathic thrombocytopenic purpura (ITP).[3] Mothers with ITP, myasthenia gravis, and/or systemic lupus erythematosus (SLE) with SSA-Ro-SSB/La immunity may transfer antibodies to their fetus, resulting in neonatal disease.[4-7] Allogeneic stem cell transplantation from donors with autoimmune disease may also transfer the disease to recipients.[8-13]

## Theories of tolerance

Clinical tolerance is failure of an organism to reject an antigen or tissue without use of immune-suppressive medications but with intact normal rejection of third-party or foreign antigens. The oldest theory of tolerance, and now viewed as orthodoxy, is clonal selection of lymphocyte repertoires.[14] Self-reactive lymphocytes are deleted and not allowed to mature. Clonal selection as an explanation for tolerance was first proposed by Burnet[15] in 1957 in regards to antibody formation and self-recognition and non–self-recognition. Subsequently, this concept was extended to selection of T cells by deletion of autoreactive clones within the thymus.[16-21] T-cell precursors emigrate from the marrow to the thymus. In the thymus, if self-antigen of sufficient concentration and affinity for their specific T-cell receptor (TCR) repertoires is present, the T cells undergo apoptosis (deletion) or anergy (functional silencing).[22-25] Because lymphocyte progenitors are continually generated from HSCs, clonal selection would have to be an ongoing process occurring throughout life.

Thymic editing includes not only negative selection to delete self-reactive clones but also positive selection to allow maturation of self-reactive clones.[17,26] If a particular TCR fails to engage a major histocompatibility complex (MHC) peptide/complex, or binds it too tightly, it undergoes apoptosis. If it recognizes an MHC/peptide complex with moderate avidity, it is positively selected and undergoes further maturation. The avidity (concentration and binding affinity) of an MHC/peptide complex appears to play a role in positive versus negative selection of T lymphocytes.[27,28] Intrathymic selection and anergy as a mechanism of maintaining tolerance of autoreactive repertoires was, therefore, amended by theories concerning peripheral tolerance.[29,30]

Mechanisms of peripheral tolerance revolve, in part, around the 2-signal hypothesis of self-discrimination and non–self-discrimination introduced by Bretscher and Cohn[31] in 1970. T cells, positively selected within the thymus, remain anergic unless antigen is presented with a second signal (ie, a costimulatory signal). Basically, antigen presentation to a T cell without costimulation maintains anergy, whereas TCR engagement of antigen combined with costimulation results in T-cell activation.[32-35]

The traditional costimulatory molecule for T-cell activation is CD28, a ligand for B7-1 (CD80), and B7-2 (CD86) receptors on T cells.[36] CD28 binding increases transcription of interleukin 2 (IL-2).[35,37] A variety of other molecules, including CD40L, inducible costimulator (ICOS), and various adhesion molecules, also provide secondary or tertiary signals to facilitate T-cell activation.[38-43] Requirement of costimulation for activation may place some constraints on peripheral sites for cellular activation. Antigen-presenting cells (APCs) that express costimulatory molecules are

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localized within secondary lymphoid tissues (spleen and draining lymph nodes). Transfer of antigen by immune cells to secondary lymphoid regions may be important to induce T-cell activation.[44] For example, allogeneic tissue grafts are not rejected in mice that lack secondary lymphoid tissue.[45]

Besides the requirement for costimulation, a variety of mechanisms maintain peripheral tolerance. Some of these mechanisms are similar to intrathymic tolerance but occur in the periphery, including peripheral T-cell deletion and/or anergy induced by T-cell interaction with parenchymal cells.[46,47] Other checks to maintain peripheral tolerance include activation-induced cell death,[48] suppressor or regulatory cells,[49-51] and peripheral antigen avidity (ie, antigen persistence, concentration, and affinity).[52,53] Theories on peripheral tolerance explain how a T-cell repertoire selected intrathymically for reactivity to self maintains peripheral tolerance. A further extension of tolerance to what has been termed the "danger signal" explains the context in which costimulation arises.[54]

The danger metaphor proposed by Matzinger[54] involves the use of the innate immune system (neutrophils, natural killer cells, and macrophages) to break peripheral tolerance. T-cell–mediated immunity, known as adaptive immunity, is an evolutionary development of vertebrates.[55] Adaptive immunity involves the rearrangement of a limited number of germ line genes to produce a highly diversified repertoire of approximately $10^{14}$ to $10^{18}$ somatically mutated T-cell (immunoglobulinlike) receptors and B-cell immunoglobulin receptors. These T cells undergo deletion and anergy within the thymus. However, the innate immune system does not have pathogen-receptor repertoire diversity.[56] Response to infection is intrinsic to a limited number of germ-line receptor genes that recognize pathogen-specific molecular patterns. These patterns include receptors for conserved pathogen structures like lipopolysaccharides, mannans, bacterial DNA, and lipoteichoic acids. Receptor-mediated phagocytosis of pathogens by macrophages leads to release of proinflammatory cytokines and expression of costimulation molecules, along with MHC presentation of pathogen-derived peptides, leading to T-cell activation. Thus, pathogen stimulation of innate immunity can lead to activation of the adaptive immune system.[57-59]

In animal models, active immunization with self-epitopes requires an adjuvant (immune stimulant) to break tolerance. Adjuvant is often nothing more than homogenized pathogens such as mycobacterium, which provides the danger signal for activation of innate APCs such as macrophages. Presentation of coinjected self-proteins by adjuvant-activated APCs initiates antigen-specific autoreactive T cells. Once activated to self by innate immunity, how is the adaptive immune system prevented from causing autoimmune disease? This question may be approached by viewing the immune system as dynamic and constantly fluctuating.

In all prior theories of tolerance, lymphocytes are viewed as responding or not responding, like a light switch that is on or off. The perturbation theory postulated by Grossman and Singer[60] and Grossman and Paul[61,62] proposes that lymphocytes are dynamically tuned much like a rheostat dims or brightens a room. Lymphocytes selected intrathymically may maintain a steady tone by repeated interaction with peripheral tissue. It is the sudden change in dynamic homeostasis that is perceived as a perturbation. By analogy, blood is always dynamically fluctuating between clotting and lysis. In steady state, blood may be erroneously perceived as static. The immune system may also be dynamically fluctuating between autoimmunity and tolerance in a dynamic steady state not readily appreciated. A steady state that may be controlled by clonal selection, activation, feedback inhibition, and intracellular receptor and signal transduction tuning. It is conceivable, but unproven, that immune ablation followed by infusion of hematopoietic stem cells (HSCs) may "reset the immune rheostat."

## Breaking tolerance by environmental exposure

All processes involving tolerance, even deletion, are ongoing recurring events and may be broken. Both central and peripheral T-cell tolerance may be broken by environmental exposure. Classic agents capable of breaking tolerance include drugs and infections.[63-65]

### Drug-induced autoimmunity

Numerous drugs may cause autoimmunity by affecting thymic TCR antigen interaction or TCR signal events. A common drug associated with lupuslike manifestations is procainamide.[66-68] When the metabolite procainamide-hydroxylamine is injected into the thymus of an animal or added to primary thymic organ cultures, chromatin-reactive T cells emerge.[66] Procainamide-hydroxylamine may alter the avidity of TCRs for self-antigen, preventing deletion of some autoreactive T-cell repertoires.[68] Cyclosporine is an immunosuppressive medication that inhibits TCR-mediated signaling. By inhibiting peripheral T-cell activation, cyclosporine suppresses autoimmunity but by interference with thymic TCR signaling may also inhibit thymic deletion of autoreactive T cells,[69-72] causing a T-cell autoimmune sclerodermalike disease termed syngeneic graft versus host disease (GVHD).[72]

Drug-induced disruption of central tolerance implies existence of a functional thymus throughout adulthood. By using the membrane protein CD45 to differentiate naive (CD45 RA) from memory (CD45RO) T cells, thymic-dependent T-cell production appears to diminish markedly after puberty, presumably because of thymic atrophy. If the thymus involutes, new adult T cells would then be derived exclusively from peripheral expansion of existing memory cells. However, with the advent of newer DNA assays, the accuracy of differentiation between naive and memory T cells by CD45 has been questioned.[73-75]

During TCR thymic development, rearrangement of TCR genes leads to excision of circular DNA termed T-cell receptor rearrangement excision circles (TRECs).[73] TRECs are episomal, unique to T cells, and do not duplicate during mitosis. Because TCR rearrangement occurs during thymic development, TRECs may be used as a marker for recent thymic emigrants. In the early post–hematopoietic stem cell transplantation (HSCT) period, there is a substantial increase in peripheral blood TREC-positive T cells.[74] Although an inverse correlation exists between age and TREC production after HSCT, TREC numbers increased in all age groups. Therefore, thymic-dependent generation of T cells occurs in all ages. A drug or environmental-related disruption of thymic tolerance, which alters TCR antigen avidity or TCR cytoplasmic or nuclear signaling events, may allow escape of autoreactive lymphocytes. Once in the periphery, long-lived autoreactive cells could cause a persistent autoimmune disease.

### Infection-induced autoimmunity

An infectious agent has been associated with virtually every autoimmune disease, including diabetes mellitus,[76-79] ankylosing spondylitis,[80] multiple sclerosis (MS),[81-86] myocarditis,[87-89] rheumatoid arthritis (RA),[90-96] and SLE.[97] These associations are suggested by epidemiologic studies and serology that connect disease onset or

flare to various infectious agents, cross-reaction of virus or pathogen epitopes and self-proteins, and occasional isolation of an infectious agent in affected tissue.

An infection could precipitate an autoimmune disease by breaking self-tolerance through molecular mimicry,[98,99] determinant or epitope spreading,[100,101] or bystander activation.[102] Molecular mimicry is the capacity of a lymphocyte activated to an infectious pathogen to cross-react with a similar host determinant. Because memory lymphocytes are long lived, the infectious agent that initiated molecular mimicry to self does not need to persist for autoimmunity to occur. This situation may be one reason for difficulty in proving an infectious etiology for autoimmune disorders. Bystander activation arises when activation of T cells specific for antigen X occurs during an immune response against a nonhomologous antigen Y. In contrast, molecular mimicry is targeted toward self-peptides homologous to the initiating determinant on a viral or other infectious agent. Immunization with adjuvant and peptide is an example of bystander activation to the coinjected nonhomologous peptide.[103]

Infection-related inflammation is associated with tissue destruction and presentation of self-epitopes, as well as up-regulation of APC costimulatory molecules that may also lead to bystander activation of T cells to self-determinants. Theiler murine encephalomyelitis virus (TMEV)–induced demyelination, an autoimmune demyelinating disease that mimics MS, is an example of viral-induced bystander activation.[104] TMEV is a picornavirus (small RNA virus) that infects gray matter neurons but, through bystander activation of the immune system, leads to an autoimmune-demyelinating white matter disease.[105]

Superantigens may also cause bystander activation. Superantigens are bacterial, mycoplasma, or viral proteins that activate polyclonal groups of T cells.[106-112] Polyclonal activation arises by cross-linking the side of a MHC molecule to the Vβ portion of a TCR. Superantigen binding occurs outside the MHC peptide–binding groove and outside the TCR CDR3 antigen-specific recognition site. Activation by superantigen results in overexpansion and/or deletion of entire Vβ families, resulting in skewing of the T-cell repertoire. Superantigen activation of T cells has been suggested to initiate or cause a flare of various autoimmune diseases, including myocarditis, diabetes, MS, and psoriasis.[107]

Once molecular mimicry, bystander activation, or superantigens initiate an autoimmune disease, the immune response spreads over time to epitopes that are distinct and non–cross-reactive to the inducing epitope, a phenomenon termed determinant or epitope spreading.[113] Epitope spreading has been documented for both T- and B-cell immune responses. A hierarchical order of epitope spreading occurs according to immune dominance of the epitope. Determinant spreading may occur to different regions on the same protein (intramolecular epitope spread) or to a protein distinct from the protein containing the disease-initiating epitope (intermolecular epitope spreading). Temporal spreading of immune responses to other epitopes has been demonstrated in numerous animal autoimmune disorders, including experimental autoimmune encephalomyelitis (EAE),[114] diabetes in nonobese diabetic (NOD) mice,[115] and experimental autoimmune myasthenia gravis.[116] Determinant spreading is suspected to be associated with several human autoimmune diseases, including MS,[117] SLE,[118] bullous skin diseases,[119] myasthenia gravis,[120] diabetes,[121,122] and chronic rejection of organ allografts.[123-125]

The mechanism of epitope spreading may be related to costimulation, because in some models blocking CD28/B7 costimulation may prevent epitope spreading.[100] Whatever the mechanism, epitope spreading makes it difficult to retrospectively determine the inducing epitope or antigen. Effectiveness of targeted immune interventions directed against one TCR or epitope may be limited by the phenomenon of epitope spreading.

## Genetic susceptibility to breaking tolerance

### MHC autoimmune-associated genes

MHC antigens were initially referred to as tissue transplantation antigens. They were discovered, as the name implies (major histocompatability complex), to have a major role in rejection of transplanted organs. As later discovered by Zinkernagel and Doherty,[126] the MHCs are peptide-presenting molecules resulting in MHC/peptide restriction for T-cell recognition.[127] It is not, therefore, surprising that many autoimmune diseases are associated with particular MHC genotypes.

Numerous suspected autoimmune disorders (such as MS, RA, spondyloarthropathies, diabetes, myasthenia gravis, Crohn disease, primary biliary cirrhosis, autoimmune hepatitis, SLE, vasculitis, pemphigus vulgaris, and Sjögren syndrome) are associated with MHC alleles.[128] Because combined MHC/peptide presentation is essential for T-cell activation, a MHC association may be indirect evidence for an immune pathogenesis. RA-prone MHC alleles, their frequencies vary for different ethnic groups, share a similar amino acid epitope sequence (LLEQKRAA or LLEQRRAA) encoded by codons 67 to 74.[129-131] The HLA sequence 67 to 74 is a HLA contact site for both peptide and TCR binding. This finding suggests HLA presentation of a common infectious or self-antigen to T cells is involved in the pathogenesis of RA. Spondyloarthropathies are linked with only some molecular subtypes of HLA-B27.[132] Similar to RA, peptide-binding differences may explain differences in disease susceptibility. HLA-B27 may even present its own B27-derived peptides. In which case, the putative arthritogenic peptide may be a component of the HLA-B27 molecule.

The autoimmune etiology for scleroderma is questionable because of poor response to immune suppressive medications. Similarly, scleroderma also has a relatively weak MHC association that may indicate only partial immune pathogenesis or weak linkage of scleroderma genes to MHC alleles or the absence of an autoimmune etiology.[133] Although MHC genes correlate with autoimmune disease susceptibility, most patients with disease-associated MHC genes remain disease free throughout their lifespan. Environment and/or non-MHC genes must, therefore, contribute toward development of disease.

### Non-MHC autoimmune genes

Multiple non-MHC genes that regulate cell proliferation (oncogenes), cell signaling (tyrosinases), immune response (costimulatory molecules, interleukins, and cytokines), and apoptosis (fas) may play a role in development of autoimmunity.[134] Analysis of the diabetic-prone NOD mouse has revealed at least 18 insulin-dependent diabetes prone genes.[135] SLE occurs in various strains of mice, including Murthy Roth lymphoproliferative (MRL/lpr) mice and New Zealand Black X New Zealand White F1 hybrid (NZB/NZW) mice.[136] Various mating crosses of lupus-prone mice, as well as backcrosses to normal mice, have linked murine lupus to 38 different genomic loci.[137] Some loci are associated with glomerulonephritis, others with vasculitis, some with anti-ds DNA, some with antichromatin antibody, some with lymphoproliferation, and others with splenomegaly. No single gene is sufficient to cause

disease. Various combinations of SLE-prone genes among different patients may explain why patients with SLE can have highly variable organ involvement and clinical symptoms. Collagen-induced arthritis in rats is a model for RA and is induced by injection of collagen and adjuvant.[138,139] At least 14 genomic intervals or collagen-induced arthritis (CIA) loci are associated with collagen-induced arthritis.[140,141]

Although autoimmunity involves MHC and numerous non-MHC genes, environmental interactions with these genes are essential to manifest disease. Approximately two thirds of syngeneic twins with MS, RA, SLE, or type I diabetes are discordant for clinical disease.[142] Although a concordance rate of 33% is much higher than the general population, it remains significantly below a predetermined dominant Mendelian penetrance of 100% and suggests that environmental factors continue to have a significant role in polygenic autoimmune diseases.

## Induction of tolerance by immune ablation and autologous stem cell transplantation

### Animal models and anecdotal case reports

Animal autoimmune diseases that are induced by immunization with adjuvant or self-peptide and adjuvant may be ameliorated by syngeneic or pseudo-autologous HSCT.[143-155]

Immunization with adjuvant and either myelin basic protein or proteolipid protein peptides induces a T-cell–mediated demyelinating disease, EAE, that, depending on the animal model, may be monophasic, relapsing-remitting with secondary progression, or progressive from onset. EAE in Swiss Jackson Laboratory/Jackson (SJL/J) mice is a relapsing, remitting, and secondarily progressive disease. Several investigators have demonstrated cure, decreased relapse rates, or decreased disease severity in EAE animals undergoing syngeneic HSCT.[146,149-151] Because of the expense of long-term animal housing, most experiments in EAE are performed before disease onset to abort disease initiation or shortly after disease onset to ameliorate its course. It is unlikely that such experiments are applicable to patients with a long duration of MS with accumulated disease burden and tissue damage. Syngeneic HSCT performed in mice with chronic EAE, unlike the results in acute EAE, failed to demonstrate neurologic improvement.[146] Histologic analysis revealed chronic scarring with glial proliferation that is unaffected by HSCT.[146] To be effective as therapy for EAE, HSCT needs to be performed early in the disease course during its inflammatory stage and before accumulation of disease burden. A principle that may also be important for MS.

Murine bone marrow transplantations are performed by killing and removing the femur from the donor and using a syringe to flush out the marrow cells. It is technically difficult and inhumane to perform a murine autologous transplantation because the surviving recipient's legs would have to be amputated. However, marrow could be harvested from a syngeneic donor in the same active stage of EAE as the recipient, referred to as a pseudoautologous transplant. HSCT of EAE using pseudoautologous donors suggests that infused lymphocytes contaminating the graft may contribute to relapse.[147] This suggestion indicates that lymphocyte depletion of grafts may be important in decreasing posttransplantation relapse after autologous HSCT.

Besides immunization with myelin peptides, demyelinating central nervous system disease may be induced with viruses such as TMEV.[156] Autologous HSCT of TMEV-induced demyelinating disease causes a high mortality from viral superinfection of the central nervous system during the postconditioning pancytopenic period.[156] Autoimmune disease mediated by an infectious agent can be rapidly fatal after autologous HSCT but only if the infectious agent is still present at the time of transplantation.

Several other environmentally induced animal autoimmune diseases are improved or cured by syngeneic HSCT. These diseases include experimental autoimmune myasthenia gravis,[153] adjuvant arthritis [154,155] and collagen-induced arthritis.[145] Encouraging results of syngeneic and pseudoautologous HSCT in animal-induced autoimmunity supported the design of autologous and syngeneic HSCT trials in patients with severe autoimmune disorders.

Anecdotal case reports of patients with a coincidental autoimmune disease and a malignancy provided further support and rationale for trial design.[157-166] Refractory autoimmune diseases entered remission sometimes for several years. Because the indication for transplantation was a malignancy, and the outcome was reported retrospectively, in most cases a detailed pretransplantation evaluation by a rheumatologist or neurologist is missing. The autografts were usually not purged of lymphocytes, and the transplantations were not tailored as therapy for an autoimmune disease. Duration of response appeared shorter for RA compared with SLE. Too few patients have been reported for other autoimmune diseases, and long-term results of response to treatment in those that relapse, as well as duration of remission in those who had not relapsed, remain unknown.

### Mobilization of HSCs

Collection of stem cells from patients with autoimmune diseases is based on methods already established for patients with nonautoimmune disorders. The complications and risks of the procedure appear greater in patients with autoimmune disease and are specific for the autoimmune disease and involved organ system.[167] The most common peripheral blood stem cell (PBSC) mobilization regimens are single-agent granulocyte colony-stimulating factor (G-CSF) or cyclophosphamide and G-CSF.

Flares of MS and RA have occurred while patients were taking G-CSF for mobilization.[167,168] MS flares have resulted in serious and irreversible neurologic deterioration. G-CSF–related flares of RA are relatively mild, being manifest as a transient increase in the number of swollen or tender joints that resolves with or without an increase in corticosteroid dose.[167] The only complications of G-CSF PBSC mobilization in patients with scleroderma are transient telangiectasia that spontaneously resolves.[167] In other diseases, such as SLE, there exists virtually no data on PBSC with G-CSF as a single agent. The simultaneous administration of G-CSF and steroids has been used in a limited number of patients without disease exacerbation.[169]

To prevent G-CSF–related disease flare, combined cyclophosphamide and G-CSF (Cy/G-CSF) may be used for mobilization. However, combined Cy/G-CSF PBSC mobilization has been complicated by neutropenic-related infection and disease-specific fatal visceral organ toxicity.[167] Infections with opportunistic organisms may be more common in patients who have been on high-dose corticosteroids for prolonged intervals, such as patients with refractory SLE. Scleroderma patients with cardiac and/or pulmonary involvement undergoing PBSC with 4.0 g/m$^2$ cyclophosphamide have succumbed to cardiac arrest and/or pulmonary alveolar hemorrhage.[167] No significant regimen-related organ damage has been reported at doses of 2.0 g/m$^2$ or for doses of 4.0 g/m$^2$ in nonscleroderma patients. This finding emphasizes the importance

of adjusting the mobilization regimen based on disease and organ involvement for the minimum mobilization-related morbidity.

Although cyclophosphamide-based mobilization is generally associated with more toxicity from infection or organ damage, autoimmune diseases are generally ameliorated by the immune suppressive effects of cyclophosphamide.[167] The duration of improvement from cyclophosphamide-based PBSC mobilization is unknown because most patients proceed within a relatively short time interval from mobilization to HSCT. As an exception, in at least one autoimmune disease (Evans syndrome), cyclophosphamide-based PBSC resulted in rapid and fatal acceleration of disease activity.[170] This acceleration was attributed to a rapid cyclophosphamide-induced suppression of otherwise compensatory and accelerated hematopoiesis in the presence of persistent peripheral destruction from residual immunoglobulins against red blood cells and platelets.

There is no single optimal mobilization regimen for PBSC in patients with autoimmune disease. The PBSC method should be individualized for the disease and organ system involved. Newer mobilizing agents such as stem cell factor, thrombopoietin, chemokines, and/or high-dose corticosteroids and G-CSF need to be evaluated to collect progenitor stem cells with minimum mobilization-related morbidity.

After collection of progenitor cells, most but not all centers perform ex vivo lymphocyte depletion.[167] Because the existence or identity of suppressor cells remains vague, graft depletion techniques are nonspecific without attempts at conserving regulatory cells. Positive enrichment for $CD34^+$ cells has been performed by using either CEPRATE (CellPro, Bothel, WA), Isolex (Nexel, Irvine, CA), or CliniMACS (Miltenyi, Bergish Gladbach, Germany) cell separation systems. Negative selection was performed with T-cell antibodies by e-rosette or Nexel Isolex CD4/CD8 selection.

Insufficient clinical data are currently available to compare an unmanipulated versus a T-cell–depleted graft in terms of disease response or relapse. Aggressive lymphocyte depletion may adversely affect immune reconstitution against pathogens, increasing the risk of serious posttransplantation opportunistic infections such as cytomegalovirus, fungemia, *Pneumocystis carinii* pneumonia, or Epstein-Barr virus posttransplantation lymphoproliferative disease (PTLD).

### Conditioning regimens and the role of immunosuppressive versus myeloablative conditioning for reinduction of self-tolerance

The first convincing evidence that intense immunosuppression may cure life-threatening autoimmune diseases was obtained in a patient with mixed cryoglobulinemia in end-stage renal failure with a cryocrit level of 60%.[171] In the early 1970s, a patient with monoclonal immunoglobulin (Ig)M and polyclonal IgG was treated with a combination of cyclophosphamide and azathioprine. Treatment was complicated by lymphocytopenia and sepsis because of neutropenia, but the patient recovered with no stem cell support. After recovery, renal function normalized in parallel with elimination of the cryoglobulinemia, and the patient is alive and disease free for more than 25 years.[171] This case represents the longest observation of a patient with reinduced self-tolerance after elimination of self-reactive lymphocytes and reestablishment of immunity from uncommitted stem cells.

Brodsky et al[172] extended this early observation by treating a variety of autoimmune diseases with high-dose cyclophosphamide (200 mg/kg) without HSC infusion.[172] For some autoimmune diseases such as SLE, early results from high-dose cyclophosphamide without stem cell support are encouraging. Although the response rate is high, depending on disease, relapse is common. With the exception of some diseases such as SLE, a more intense and myeloablative regimen with stem cell support may be required for durable responses. Infusion of mobilized HSCs shortens the duration of neutropenia by 5 to 7 days, theoretically decreasing the risk of serious infections. Ex vivo expansion of HSCs before infusion may completely eliminate neutropenic-related infections. For these reasons, a trial that randomized between cyclophosphamide with or without stem cell support is not currently being planned, and the rest of this review will be devoted to immune suppression with HSC support.

Ideally, the conditioning regimen should be able to eliminate immune cells without neutropenia. Such a regimen does not exist. The more immune ablative a regimen becomes, the more likely it is to be myeloablative and require stem cell support for reconstituting hematopoiesis. The conditioning regimens being used in autoimmune transplantations were empirically developed for use in malignancies. Autoimmune conditioning regimens include cyclophosphamide (Cy)[173-177]; cyclophosphamide and polyclonal antilymphocyte antibodies such as antithymocyte globulin (ATG) or humanized monoclonal rat antihuman CD52 (Campath-1H) antibodies (Cy/ATG or Cy/Campath-1H, respectively)[178-188]; carmustine, etoposide, cytarabine, and melphalan (BEAM) [189-192]; cyclophosphamide and total body irradiation (Cy/TBI)[193]; cyclophosphamide, TBI, and antithymocyte globulin (Cy/TBI/ATG)[194,195]; busulfan and cyclophosphamide (Bu/Cy)[196,197]; busulfan, cyclophosphamide, and ATG (Bu/Cy/ATG)[198]; cyclophosphamide and thiotepa (Cy/TT)[199,200]; and fludarabine-based regimens.

Cy or Cy/ATG is the most common conditioning regimen used for HSCT of SLE.[181-184,188] Pulse cyclophosphamide (500-1000 $mg/m^2$) is a standard treatment for SLE. It is, therefore, reasonable to escalate cyclophosphamide to transplantation doses as the conditioning regimen for SLE. To avoid cardiac injury, transplantation doses of cyclophosphamide are limited to 200 mg/kg usually divided into 50 mg/kg per day. Cyclophosphamide is often used to mobilize stem cells before HSCT at doses of 2.0 to 4.0 $g/m^2$. If cyclophosphamide is used in both the mobilizing and conditioning regimen, either the conditioning regimen dose may be decreased or the time interval between mobilization and HSCT may be delayed by several weeks to minimize the risk of cardiac toxicity from total cyclophosphamide dose. When the conditioning dose of cyclophosphamide is decreased, some centers add another agent such as thiotepa.[199,200] Most patients with SLE eligible for HSCT are corticosteroid dependent and markedly cushingoid. There is a marked discrepancy between ideal and actual weight in terms of calculating cyclophosphamide dose. For safety reasons, in cushingoid patients, the dose is generally based on ideal or an adjusted ideal rather than actual weight.

Cy and Cy/ATG are conditioning regimens for scleroderma[176,187,188] and RA.[173-175,180] High-dose cyclophosphamide may be associated with high cardiopulmonary mortality in patients with scleroderma.[167] Volume shifts and infections that stress cardiovascular reserve are the likely culprit of HSCT-related cardiopulmonary collapse in scleroderma-associated pulmonary artery hypertension. In RA, organ function is generally normal, and cyclophosphamide-related toxicity is less problematic. The toxicity of a conditioning regimen, therefore, depends on the disease and disease-related organ dysfunction.

Bu/Cy regimens have been used in a limited number of HSCTs for MS[197] and RA.[196] Busulfan is fat soluble and readily crosses the

blood-brain barrier to the site of MS plaques. Busulfan is administered orally with variability in absorption and first-pass hepatic metabolism. Busulfex is an intravenous formulation that gives more uniform and less toxic serum levels. For RA, it may be equally important for efficacy that the conditioning regimen target not only lymphocytes but also synovial macrophages. Theoretically, HSCT results may be improved in RA by adding a more effective antimacrophage agent such as busulfan to a cyclophosphamide-based regimen.[201] There are special concerns about the use of Bu/Cy in RA and MS. Patients with RA may have disease-related interstitial pneumonitis with little reserve for busulfan-related lung injury. The effects of alkylating agents on demyelinated neurons are unknown. In MS, the neurotoxicity of high-dose alkylating-based conditioning regimens remains unknown.

BEAM and Cy/TBI are common lymphoma regimens being used to treat MS.[189-191,193] TBI was selected because, unlike most agents, radiation readily crosses the blood-brain barrier. To avoid TBI-related pulmonary injury, radiation is generally given in the anteroposterior and posteroanterior position with 50% lung blocks with full dose to the mediastinal lymph nodes and spinal cord. A comparison of BEAM versus Cy/TBI regimen-related toxicity has not been performed. In general, TBI regimens are not used in RA because trials of nonmyeloablative total nodal irradiation in RA were associated with unexpected late complications such as myelodysplasia.[202]

Cy/TBI/ATG has been used as a conditioning regimen in the United States for scleroderma[195] and MS,[169] and in Europe for juvenile chronic arthritis (JCA).[194] For patients with pulmonary scleroderma, TBI without lung shielding has been associated with lethal pulmonary deterioration.[195] If attenuated with partial lung shields, TBI-related scleroderma lung injury appears less likely. Cy/TBI/ATG has been associated with lethal PTLD.[358] The investigators attributed PTLD to use of high-dose rabbit ATG. Lower and less immune-suppressive doses of rabbit ATG or the use of horse ATG has not been reported to cause PTLD in autoimmune diseases.

Independent of the conditioning regimen (Cy or Cy/TBI/ATG), when combined with aggressive T-cell depletion, HSCT in JCA has been complicated by lethal macrophage activation syndrome (MAS), manifesting as fever, lymphadenopathy, hepatosplenomegaly, and disseminated intravascular coagulation.[186] MAS is a reactive hematophagocytic lymphohistiocytosis and has been associated with JCA independent of HSCT.[203] The diagnosis is confirmed on bone marrow aspirate by macrophages (or histiocytes) actively phagocytosing hematopoietic cells and may arise from immune dysregulation perhaps in response to viral infections. To date, posttransplantation MAS appears to be a complication unique to JCA.

No reports exist of late regimen-related organ toxicity from HSCT in autoimmune diseases. All patients need to be warned of infertility and of regimen-specific late toxicities such as cataracts from TBI. Late malignancies are also possible.[204] Similar to mobilization regimens, conditioning regimens must be uniquely designed for the disease, organ impairment, disease-specific infection susceptibility, and extent of prior immune suppressive medication–related infectious risk to ensure minimum regimen-related mortality.

### Mortality

Transplantation-related mortality (TRM) for all autoimmune diseases has been reported to be 8.6%.[205] TRM is disease specific, in order of highest to lowest TRM: scleroderma, SLE, MS, and RA. This mortality is higher than expected because of phase 1 trials that selected patients with advanced end-organ dysfunction and/or active and refractory disease. Judicious selection of patients earlier in disease or in remission, but with a high risk of relapse or further progression, will diminish TRM. Variability in TRM based on the center performing the transplant, also known as the center effect,[206] may be occurring for autoimmune diseases. Many factors affect TRM, including patient selection, supportive care, conditioning regimen, degree of lymphocyte depletion of the graft, use of disease-specific versus generic protocols, and so forth. A lower mortality in centers dedicated to autoimmune HSCTs may be obscured within the variability of multicenter registry data.

### Posttransplantation immunization

After HSCT, a patient's titer from prior immunizations (eg, diphtheria, measles, tetanus, hepatitis B, etc) is often low or undetectable. As discussed in the "Breaking tolerance by environmental exposure" section, immunization could, theoretically, reinduce autoimmune disease. The risk of relapse may vary according to the type of immunization. For example, there was concern that onset and flare of MS may be associated with hepatitis B vaccination, although recent studies have shown no association.[207] Although the risk of infection-related mortality or infection-induced autoimmunity in a nonimmunized individual probably outweighs any theoretical risk of immunization-induced disease relapse, guidelines on posttransplantation vaccination in autoimmunity have yet to be written.

## Specific diseases

MS, SLE, RA, and scleroderma will be discussed further because phase 3 autologous HSCT trials are being prepared in these diseases. In Europe, the European Bone Marrow Transplant/European League Against Rheumatism (EBMT/EULAR) autoimmune committee is designing these trials. In the United States, the trials are funded by the National Institutes of Health and are being designed by disease-specific working groups composed of transplant physicians, rheumatologists, and neurologists.

### Autologous HSCT for MS

MS is a relatively common North American and European disease with a prevalence of approximately 1 in 1000 people.[208] It is at onset an immune-mediated disease confined to the central nervous system. The disease is characterized by a variable course.[209-211] Patterns are (1) relapsing-remitting MS defined as relapsing disease without progression between relapses with or without residual neurologic deficits from each relapse, (2) secondary progressive MS defined as continuous (often insidious and steady) neurologic deterioration with or without superimposed relapses after an initial relapsing-remitting course, and (3) primary progressive MS defined as steady continuous deterioration from onset. At onset, approximately 15% of the cases are primary progressive and 85% are relapsing-remitting.[209-211] Within 10 years, 50% of relapsing-remitting cases become secondary progressive, and by 25 years, 90% have progressive disease. Relapse frequency in the first year of diagnosis influences time interval to disability.[209-211] The median time to difficulty ambulating without unilateral assistance (an extended disability status score [EDSS] of 6.0) is 7 years for 5 or more relapses; 13 years for 2 to 4 relapses; and 18 years for 1 to 2 relapses.

Accepted immune-modulating agents for MS are interferon beta (Avonex, Betaseron)[212-216] or Copaxone (copolymer 1 or glatiramar

acetate)[217,218] known as ABC therapy. Avonex and Betaseron are different formulations of interferon-β and Copaxone is an oral mixture of random peptide sequences containing L-glutamate, L-lysine, L-alanine, and L-tyrosine, thought to mimic myelin peptides. The ABCs of MS therapy are approved for relapsing-remitting disease and, although not approved by the U.S. Food and Drug Administration (FDA), are often used for progressive forms of MS. The immune suppressive chemotherapy drug mitoxantrone received FDA approval for secondary progressive and progressive-relapsing MS.[219,220] The need for new interventions in MS is evident from the desperation of patients who in some studies have a higher suicide rate compared with the general population.[221]

Natural history magnetic resonance imaging (MRI) studies have demonstrated that neurologic progression can continue despite lack of new demyelinating events on MRI.[222,223] Although early relapse frequency within the first year of diagnosis appears to correlate with onset of late disability, Confavreux et al[224] reported that relapse frequency in disease of longer duration and EDSS scores more than 4.0 do not correlate with disability. This finding indicates that treatment designed to prevent relapses (ie, immune-modulating therapy) used late in disease is probably not adequate to prevent progressive disability. Demyelination alone does not adequately explain the progressive disability that occurs in patients with progressive MS. Yet, the most important therapeutic goal is to prevent disability and maintain neurologic function. An evolving amount of literature on MS supports the concept that, although initially an inflammatory demyelinating disease, MS transitions into or is also an axonal degenerative disease.[225-228]

HSCT for MS was suggested in 1995.[229] In general, initial HSCTs were phase 1 studies and captured patients with progressive disease and high disability (EDSS) scores[189-191,230-233] (Table 1). The Thessaloniki group has reported a 3-year progression-free survival for primary progressive MS (39%), which appears significantly lower than for secondary progressive (92%).[190] In an Italian study, Mancardi et al[231] reported 10 subjects undergoing HSCT followed with a frequent MRI protocol who demonstrated lack of enhancing lesions and accumulation of T2 burden of disease over an observation period of 4 to 30 months. A second study with a 5-year follow-up has noted a discordant response between MRI and clinical results.[230] Some patients had clinical progression of disability, defined as an increase in the EDSS by one or more points but no new attacks or change on MRI in terms of T2 disease burden. The patients whose EDSS increased despite lack of MRI changes had significant pretransplantation disabilities (EDSS of 7.0 to 8.5). Although longer follow-up is necessary, it appears that HSCT slows or halts acute attacks and further immune-mediated demyelination but not progressive disability, especially in disease of increasing duration or higher disability scores.

Two possible phase 3 MS trial designs are being proposed to run simultaneously. For secondary progressive MS, the trial would be aimed at suppressing relapses in patients with progressive disability. Patients with accumulated baseline deficits, but still inflammatory disease, could be considered candidates. This group could include ambulatory patients with an EDSS of 3.5 to 6.0 and continued relapses (or MRI evidence of active disease) randomized between a TBI and Cy regimen with or without low-dose ATG and CD34-selected HSCs versus mitoxantrone every 3 months for 2 years. However, suppression of relapses may be insufficient to halt progressive neurologic impairment, particularly as the duration of disease and the level of disability increase.

For relapsing-remitting MS, the protocol would be aimed at suppressing relapses in patients at risk for progressive disability. Patients with relapsing-remitting disease who have failed interferon may be randomized between cyclophosphamide (200 mg/kg, with or without low-dose ATG) with CD34-selected HSC support versus best standard therapy (ie, continued interferon or interferon and adjuvant immunotherapy) (azathioprine, methotrexate, mitoxantrone, or cyclophosphamide). Because patients in this study would be earlier in the disease course, a safer conditioning regimen that does not include TBI would be indicated. Efficacy of earlier intervention in MS is supported by the Controlled High-risk Subjects Avenox Multiple Sclerosis Prevention Study (CHAMPS), in which over a 3-year interval treatment with interferon after the first clinical event significantly lowered the probability of developing clinically definite MS.[234] If early intervention before onset of progressive disease is important in preventing late disability, a safe but intense immune suppressive regimen might be indicated in patients with relapsing-remitting MS who have failed interferon.

Although the primary outcome of these trials would be progressive disability defined by the EDSS, other outcome measures would include clinical status by the Neurologic Rating Scale and Multiple Sclerosis Functional Composite, measurement of accumulated atrophy on MRI of the brain and cervical spinal cord, and potentially measures of whole brain N-acetyl-aspartate on magnetic resonance spectroscopy that reflects neuronal and axonal integrity.

### Autologous HSCT for SLE

Although studies have suggested that SLE encompasses several genetic diseases with some clinical commonalties,[235,236] the disease will be considered here as a single entity with protean clinical expressivity.[237,238] SLE has an overall prevalence that has varied from 12 to 50.8 cases per 100 000 persons.[239] Survival has improved dramatically, reaching a 90% 10-year survival and a 70% 20-year survival in the 1990s. Within

**Table 1. Results of autologous/syngeneic hematopoietic stem cell transplantation in patients with multiple sclerosis**

| Group | No. of patients* | EDSS baseline | Regimen | Progressed | Follow-up, mo, median (range) | Treatment-related deaths |
|---|---|---|---|---|---|---|
| Fassas et al[190,191] | 24 | 6.0 (4.5-8.0) | BEAM + ATG | 5/23 | 40 (21-51) | 1 |
| Burt et al[179,189,230] | 27 | 7.0 (3.0-8.5) | Cy/TBI | 4/25 | 14 (2-58) | 0 |
| Nash et al[169] | 20 | 7.0 (5.0-8.0) | Cy/TBI/ATG | 2/13 | 5 (3-24) | 1 |
| Carreras et al[232] | 10 | 6.2 (5.0-6.5) | BEAM + ATG | 2/10 | 18 (16-32) | 0 |
| Kozak et al[189] | 8 | 6.5 (6.5-7.5) | BEAM + ATG | 1/8 | 8.5 (1-16) | 0 |
| Openshaw et al[197] | 5 | 6.5 (5.5-7.5) | BU/Cy + ATG | 1/4 | 18 (17-30) | 2 |
| Mandalfino et al[233] | 1 (identical twin) | 6.5 | Cy/TBI | 0/1 | 26 | 0 |

EDSS indicates extended disability status score; BEAM, carmustine, etoposide, cytarabine, melphalan; ATG, antithymocyte globulin; Cy/TBI, cyclophosphamide and total body irradiation; and BU/Cy, busulfan and cyclophosphamide.

*Actual patient number is based on updated communication with the author and may be higher than the number reported in the reference.

the first 5 years, the main cause of death is active disease (neurologic, renal, systemic) or infection. Thereafter, causes of death tend to be infectious or cardiovascular events (strokes and/or myocardial infarction) related to hypertension and hyperglycemia/hypercholesterinemia because of chronic corticotherapy.

Three consecutive but separable levels of etiology, ethiopathogenesis, and pathogenesis have been considered for SLE.[240] It has been thought imperative to identify the specific molecular defects as the only way to design and use any novel and rational treatments.[241] In practice, however, SLE is treated with a variety of drugs, mainly immunosuppressive, that have been discussed recently.[242] Along with corticosteroids, intravenous pulse cyclophosphamide has been used in a National Institutes of Health–developed protocol specifically directed toward lupus nephropathy.[243]

At the pinnacle of the lupus iceberg, however, there are cases of refractory-relapsing ("intractable")[244] disease. For such patients, following the considerable experimental evidence discussed formerly and also on the basis of serendipitous case reports of coincidental diseases, HSCT was proposed in 1993.[245] Several cases of concomitant SLE and malignancy have been treated with HSCT and published. They include chronic myeloid leukemia/SLE,[166] non-Hodgkin lymphoma (NHL)/SLE,[157] and Hodgkin disease/SLE.[160] The first patient eventually died of his leukemia without any evidence of active SLE. In another case, the NHL did not relapse, but ITP supervened in conjunction with an anticentromere antibody.[163]

The first patient with SLE received a transplantation of her own T-cell–depleted marrow in 1996.[199] The first report on HSCT for SLE in the United States was published 1 year later in 1997.[183] There are now several fully published case reports of nonconcomitant SLE patients having undergone HSCT (Table 2).[181-184,192,199,246] All received transplantations of cyclophosphamide and G-CSF–mobilized $CD34^+$ cells, and conditioning regimens varied from Cy/TT to Cy/ATG (200 mg) to BEAM. All patients reached complete remission, but in several there was a serologic antinuclear antibody (ANA) relapse after 2 to 3 years from transplantation. In the patient with the longest posttransplantation follow-up, after 3 years of corticoid-free remission, there was a reappearance of ANA/DNA antibodies, and, after another year, there was also a mild proteinuria, which is currently being treated with a combination of corticosteroids and mycophenolate mofetil.[247]

In the most extensive single-center clinical study published to date,[181] 9 patients underwent stem cell mobilization with cyclophosphamide 2.0 g/m$^2$ and G-CSF 10 μg/kg. Two patients were excluded from transplantation because of infection (one death from disseminated mucormycosis), and 7 received autotransplantations after conditioning with cyclophosphamide (200 mg/kg), 3.0 g methylprednisolone, and 90 mg/kg equine antithymocyte globulin. All patients were seriously ill, with SLE disease activity indices of 17 to 37, including 1 case with alveolar hemorrhage and 4 with World Health Organization class III-IV glomerulonephritis and nephrotic syndrome. Lupus remained in clinical remission, and ANA became negative in all patients with 1 to 3 years of posttransplantation follow-up.

Phase 3 trials are being designed in the United States to compare autologous HSCT with a control arm. The standard of care for the control arm has generated a great deal of discussion and controversy within the working group. Potential controls could be intravenous pulse cyclophosphamide, oral cyclophosphamide, mycophenolate mofetil, or an open control of best available care. American experience with oral cyclophosphamide or mycophenolate mofetil in SLE is limited. Pulse cyclophosphamide (500-1000mg/m$^2$) has a long track record and is generally considered the standard of care. If HSCT candidates are selected for failure to pulse cyclophosphamide, it is difficult to continue failed therapy as on the control arm. One solution is to offer HSCT earlier in disease. Eligible patients with nonrenal visceral involvement need only fail corticosteroids and 3 months of pulse cyclophosphamide. For patients in whom the indication is nephritis, active disease must be present despite at least 6 cycles of monthly pulse cyclophosphamide. Enrolling patients earlier in disease who are less ill would also decrease the morbidity and mortality of HSCT. A second solution is to allow patients enrolled on the pulse cyclophosphamide arm who continue to fail to crossover to HSCT.

Numerous SLE disease activity indices exist to measure disease activity including the British Isles Lupus Assessment Group scale (BILAG),[248] Systemic Lupus Erythematosus Disease Activity Index,[249] Systemic Lupus Activity Measure,[250] and the Lupus Activity Index.[251] The index used depends on institutional and investigator familiarity. In the American phase 3 trial of HSCT for SLE, the disease activity instrument will be the BILAG. BILAG is one of the more useful instruments for characterizing disease stage because BILAG score correlates with necessity to treat and has been validated as an instrument to measure disease activity.[252,253] The evaluation is based on a 5-category classification, characterizing the degree of symptoms attributed to active lupus for 86 questions based on the patient's history, examination, and laboratory results. The 5 categories of response are the following: not present, improving, same, worse, and new. The 86 questions are grouped into the following 8 systems: general, mucocutaneous, neurologic, musculoskeletal, cardiovascular and respiratory, vasculitis, renal, and hematologic. For each of the 8 systems, a severity grade (A to E) is

**Table 2. Results of autologous hematopoietic stem cell transplantation in patients with systemic lupus erythematosus**

| Reference | No. of patients receiving transplants | Regimen | Results | Mortality |
|---|---|---|---|---|
| Marmont et al[199] | 1 | TT/Cy | Clinical remission for more than 3 y, serologic relapse | 0 |
| Burt et al[179,183] Traynor et al[181] | 9 | Cy/ATG | Clinical remission for up to 4 y, 2 relapsed at 3 y and 3.5 y, respectively | 1/12 mobilized |
| Fouillard et al[192] | 1 | BEAM | Clinical remission for 1 y; ANA negative at 6 mo but positive at 9 mo | 0 |
| Rosen et al[188] | 3 | Cy/ATG | Complete remission of active disease | 0 |
| Musso et al[185] | 1 | Cy/ATG | Posttransplantation low ANA titer and low Coombs positive at 8 mo but anti-ds DNA negative and anticardiolipin antibody negative | 0 |

TT/Cy indicates thiotepa and cyclophosphamide; Cy/ATG, cyclophosphamide and antithymocyte globulin; BEAM, carmustine, etoposide, cytarabine, melphalan; ANA, antinuclear antibody; and anti-ds, anti–double strand DNA antibody.

calculated according to the scores. The following list indicates interpretation of each of the grades for each system: A, disease is active enough to need treatment; B, disease has the potential to need treatment soon; C, disease currently does not meet grade A or B criteria; D, disease has satisfactorily resolved; and E, disease has never been involved. Because a crossover arm is tentatively planned in the American phase 3 trial, the primary endpoint will be need to treat as defined by a BILAG grade A.

### Autologous HSCT for RA

RA affects 1% of the North American population.[254] It is an immune-mediated disease that involves joint synovium with formation of an inflammatory pannus that erodes cartilage and bone.[255] The characteristic joint lesion in RA includes an increase in the numbers of both fibroblastlike and macrophagelike synoviocytes in the synovial intimal lining, infiltrating lymphocytes, plasma cells, monocytes, and macrophages. T cells comprise about 30% to 50% of synovial tissue cells. Synovial T cells have been demonstrated to have a restricted repertoire[256,257] and to react to a variety of microbial antigens[258] and self-antigens such as type II collagen epitopes.[259] Synovial macrophages produce IL-1 and tumor necrosis factor α (TNF-α).[260] RA fibroblastlike synoviocytes can proliferate in an anchorage-independent manner, escape contact inhibition,[261] aggressively invade into cartilage when coimplanted into severe combined immune deficient mice,[262] and have somatic mutations of the p53 tumor suppressor gene.[263] These complexities underscore the shortcomings of previous approaches designed to eliminate only one set of immune cells.

The most common rheumatoid symptoms are joint pain, swelling or deformity, morning stiffness, elevated sedimentation rate, and a positive rheumatoid factor. Extra-articular symptoms may occur, including rheumatoid nodules, vasculitis, and pulmonary interstitial fibrosis.[264,265] Patients with more than 20 to 30 involved joints have a 5-year mortality of 40% to 60%.[266-275] Despite newer therapeutic agents like anti-TNF drugs, about 5% to 10% of patients with RA continue to have a desperate need for better and more definitive therapies.[276] Because RA may be associated with significant morbidity, oncogene mutation, loss of synoviocyte growth inhibition, and, in some patients, high mortality, it is perhaps surprising that it was not until 1997 that the first HSCT for RA was reported from Australia[173] and the first American HSCT for RA reported in 1998.[180]

In general, the procedure has been well tolerated without mortality (Table 3). HSCT offers an almost immediate relief of symptoms. Patients become pain free, sometimes for the first time in years. Activities required for daily living, such as buttoning a shirt or combing hair, rapidly return to normal. Morning stiffness resolves, rheumatoid nodules disappear, sedimentation rate normalizes, and rheumatoid factor may disappear. Although these studies demonstrated that high-dose cyclophosphamide was well tolerated with marked improvements (American College of Rheumatology [ACR] 50 or ACR 70), a complete remission was unusual and relapse within 1 to 2 years is common.[173,175,180,277,278] There are suggestions of a dose-response effect. A dose escalation study of cyclophosphamide at 100 mg/kg revealed transient 1- to 2-month responses but at 200 mg/kg response duration increased to 18 to 20 months.[174] Too few myeloablative transplantations, for example a busulfan and cyclophosphamide regimen, have been performed to determine if durable remissions are feasible.

For an intense and expensive treatment such as HSCT to be considered for RA, sustained complete remissions or 70% improvement as defined by the ACR (ACR 70) must be achieved.[279] Several modifications are being considered, including the use of the current easily tolerated nonmyeloablative yet highly immunosuppressive regimen with posttransplantation immune modulation, eg, a TNF inhibitor, cyclosporine A, and/or methotrexate; or the use of a more intense myeloablative regimen such as busulfan and cyclophosphamide.

A European approach being proposed for phase 3 trials uses the current cyclophosphamide mobilization (2.0 to 4.0 g/m$^2$) and cyclophosphamide conditioning (200 mg/kg) with posttransplantation immune modulation. The nontransplant arm will be cyclophosphamide mobilization only followed by maintenance methotrexate (John Snowden, verbal communication, May 2001). This approach assumes that RA is not curable but is more easily controlled with conventional therapies after HSCT. Continued posttransplantation immune suppression may increase the risk of posttransplantation opportunistic infections. The Australians, rather than comparing HSCT with another therapy, are randomizing patients with RA to HSCT with or without T-cell depletion of the autograft. The American and Israeli approach is to pilot phase 1/2 autologous HSCT studies by using more intense myeloablative regimens (fludarabine plus oral busulfan or intravenous Busulfex and cyclophosphamide) in the hope of inducing more durable remissions, while simultaneously developing mini-allogeneic HSCT protocols for patients with HLA-matched siblings.

### Autologous HSCT for scleroderma

Scleroderma is a rare disorder with a prevalence of anywhere from 2 to 100 per one million people.[280] Two subsets of scleroderma are generally recognized, limited and extensive cutaneous scleroderma. Limited cutaneous scleroderma is characterized by cutaneous involvement of acral areas (hands, face, feet, forearms) but not the trunk. Limited scleroderma generally has a good prognosis. Diffuse cutaneous scleroderma is characterized by truncal and acral

**Table 3. Results of autologous hematopoietic stem cell transplantation in patients with rheumatoid arthritis**

| Reference | No. of patients | Conditioning | Comment | Mortality |
|---|---|---|---|---|
| Joske et al[173] | 1 | Cy | Marked improvement at 6 mo follow-up | 0 |
| Snowden et al[174] | 8 | Cy | Cohort I, cyclophosphamide 100 mg/kg-response for 1-2 mo<br>Cohort II, cyclophosphamide 200 mg/kg, improved for 17-19 mo | 0 |
| Burt et al[179,180] | 4 | Cy/ATG | Marked improvement up to 18 mo but 2 relapsed | 0 |
| Pavletic et al[178] | 2 | Cy/ATG | Relapsed at 5 and 7 mo | 0 |
| Durez et al[196] | 1 | BU/Cy | Remission > 10 mo | 0 |
| McColl et al[175] | 1 | Cy/ATG (identical twin) | Remission > 24 mo | 0 |
| Munro et al[278] | 1 | N/A | Marked improvement for 1 y | 0 |
| Verburg et al[277] | 12 | Cy | Marked improvement in 8/12 patients with follow-up, ranging from 7-21 mo | 0 |

Cy indicates cyclophosphamide; Cy/ATG, cyclophosphamide and antithymocyte globulin; BU/Cy, busulfan and cyclophosphamide, and N/A, not applicable.

skin involvement and early visceral (lung, renal, cardiac, gastrointestinal) involvement. For all patients with diffuse scleroderma, 5-year mortality is 25% to 30%.[281] High skin scores,[282] pulmonary, renal, or cardiac involvement is associated with a higher mortality of 40% to 50% within 5 years.[282-286]

Scleroderma is characterized by fibrosis (ie, excessive deposition of collagen in skin and visceral organs). The etiology of scleroderma is unclear, and an autoimmune pathogenesis remains controversial. Unlike MS, RA, and SLE, the MHC association is weak.[287,288] Randomized trials of D-penicillamine, interferon-α, or methotrexate either are no better than placebo or improve skin score with little beneficial effect on visceral organ function.[289-291] An exception is pulse intravenous cyclophosphamide, which appears to ameliorate scleroderma-related pulmonary alveolitis.[292] Scleroderma may be a vasculopathy, connective tissue disorder, and/or immune-mediated disease. Raynaud phenomena, nail fold capillary abnormalities, and elevated plasma von Willebrand antigen are indications of a vasculopathy with endothelial injury that may secondarily lead to ischemia and fibrosis.[293-295] Scleroderma may be a connective tissue disease. The tight skin mouse, which is an animal model for scleroderma, is a genetic connective tissue disease because of a defect in the fibrillin 1 gene.[296-298]

Support for an immune-mediated etiology include a variety of autoantibodies, including antitopoisomerase (Scl-70) antibodies,[299,300] and anticentromere antibodies.[301] Chronic GVHD is an immune-mediated disorder that is clinically and histologically similar to scleroderma.[302-304] Similar to scleroderma, chronic GVHD is associated with tissue fibrosis and is slow to respond to immune suppression. GVHD is caused by allogeneic lymphocytes, and patients with scleroderma have been reported to have an increased incidence of allogeneic hematopoietic cellular microchimerism.[305] Transplacental transfer of fetal lymphocytes to the mother may lead to mixed chimerism in postpartum females.[306] Transplacental transfer of maternal lymphocytes to the fetus may cause mixed chimerism in males and nonparous females. Similar to scleroderma, GVHD is also associated with endothelial damage and elevated von Willebrand antigen.[307] The perceived failure of immune therapies in both chronic GVHD and scleroderma may be due to neglect in recognizing or effectively treating an early inflammatory phase. Late fibrotic processes may progress and regress more slowly.

Regardless of etiology, because of its poor prognosis and lack of effective therapies, patients with scleroderma are being enrolled in HSCT protocols.[176,195] Early results indicate improved skin scores and activities of daily living but unchanged renal, cardiac, and pulmonary function. In a study of mostly European patients by using a variety of conditioning regimens, skin score generally improved with stabilization of lung function. Overall mortality was 27% because of 10% disease progression and 17% transplantation-related mortality.[308] These results suggest that more careful selection of patients earlier in disease is necessary in the design of phase 3 trials. Phase 3 randomized trials of HSCT versus monthly pulse cyclophosphamide are accruing in Europe and are being designed in the United States. The primary endpoint of these trials is overall survival.

## Induction of tolerance by allogeneic HSCT

### Animal models

Animal autoimmunelike diseases that occur spontaneously (without known precipitating infection or immunization) are not cured by a syngeneic HSCT. In fact, disease may be transferred to a normal strain of mice by HSCT from the autoimmune-prone donor.[309] Syngeneic HSCT in spontaneous-onset lupuslike disease of MRL/lpr mice resulted in only transient disease amelioration.[310] Curing a spontaneous-onset autoimmunelike disease requires allogeneic HSCT from a nonautoimmune-prone donor.[311-321] Murine spontaneous-onset lupuslike disease is cured by allogeneic HSCT from a normal donor strain.[311,314,315,317] Spontaneous-onset diabetes in NOD mice is prevented by allogeneic HSCT from a nondiabetic prone strain[316,319,321] and cured by combined pancreas and allogeneic HSCT from the same donor.[318] In fact, the "tolerizing" effect[322,323] of HSCs is best demonstrated by donor-specific organ tolerance when combining solid organ and marrow transplant from the same donor.

Donor-specific organ tolerance was initially performed by lethally irradiating animals to ablate their marrow followed by allogeneic donor bone marrow transplantation.[324-326] Although donor-specific tolerance is associated with hematopoietic chimerism, the cellular mechanism by which donor-specific tolerance arises is not fully understood.[327] Fas ligand is a surface protein that can signal other cells expressing Fas to undergo apoptosis. Fas ligand expression appears to be necessary for donor marrow to induce donor organ tolerance, because hematopoietic-induced donor-specific tolerance does not occur in Fas knockout mice.[328] Therefore, the mechanism of allogeneic HSCT-induced tolerance to solid organ grafts may be in part explained by donor-induced apoptotic deletion of graft reactive cells. It has been postulated that allogeneic HSCT may induce tolerance to autoimmune epitopes by a similar deletion of autoreactive repertoires, a phenomena termed graft versus autoimmunity (GVA).[329,330] A graft-versus-disease effect has already been established as the mechanism of remission for several hematologic malignancies, first discovered in 1981 and termed graft versus leukemia.[331,332]

A putative GVA effect is supported by experiments showing that allogeneic chimerism by using a sublethal conditioning regimen followed by allogeneic transplantation can prevent the onset of diabetes and even reverse preexisting insulitis in NOD mice, whereas the same radiation protocol without allogeneic HSC is insufficient.[333] With nonmyeloablative-conditioning regimens, spontaneous animal models of autoimmunity have been cured in the setting of mixed chimerism.[333-336] These experimental findings support low-conditioning preparative regimens for allogeneic transplantations in human autoimmune diseases.

Although in theory a GVA effect may be beneficial, the most significant toxicity of allogeneic HSCT is an immunologic reaction of donor cells against normal host tissues, a complication known as GVHD. Mini-conditioning may be associated with less GVHD compared with the more hazardous high-dose transplantation regimens. A lower GVHD risk may be due to reduced regimen-related tissue damage, decreased inflammatory cytokine release, decreased exposure of hidden tissue epitopes, and veto of alloreactive donor lymphocytes by hematopoietic cells of host origin, particularly $CD8^+$ cells.[337,338] Mini-transplantations are less likely to provide the danger signal hypothesized by Matzinger[54] that is necessary to break peripheral tolerance.

### Allogeneic HSCT in patients with autoimmune diseases

Anecdotal case reports of patients undergoing allogeneic HSCT for malignancy or aplastic anemia and a coincidental autoimmune disease have in most cases resulted in long-term remission of the autoimmune disease.[339-353] Most patients maintain remission indefinitely after discontinuation of immune-suppressive prophylaxis for

GVHD. An occasional patient has relapsed despite being chimeric (ie, 100% donor hematopoiesis). Chimeric analysis of peripheral blood for residual host hematopoiesis may, however, be falsely negative. Separation and analysis of lineage-specific subsets, such as only T cells, may reveal mixed chimerism (both donor and host cells) in only the T-cell lineage. The clinically asymptomatic donor may also have subclinical disease, such as rheumatoid factor positive, that could adoptively transfer the same disease for which the recipient received a transplant. Alternatively, because the patients are MHC matched, the donor and recipient may have similar non-MHC autoimmune genes that in the presence of host "factors," such as a persistent latent infectious agent or recurrent environmental exposure, may initiate de novo disease.

HLA-matched sibling allogeneic transplantations have already been successfully performed for some hematologic autoimmune diseases, including a case of hemolytic anemia,[354] pure red cell aplasia,[355] and Evans syndrome.[356,357] Phase 1 allogeneic HSCT trials using mini-conditioning regimens with and without lymphocyte-depleted grafts are being suggested and designed for autoimmune diseases. Just as in autologous HSCT, protocols will need to be tailored for each disease.

## Summary

HSCT of autoimmune disorders has raised new expectations, opportunities, and questions. What is the best mobilization regimen? What is the optimal conditioning regimen? Does T-cell depletion of the graft result in self-tolerance and decreased relapse, or rather result in an increased risk of infections? Can we predict candidates likely to relapse after autologous HSCT? Is relapsed disease responsive to previously refractory therapy and easier to control? Is HSCT cost effective? What is the mechanism(s) of posttransplantation remission? Which, if any, diseases may be cured by an autologous graft and which will require an allograft? Encouraging phase 1 trials have propelled this field to phase 3 trials in MS, SLE, RA, and scleroderma. Completion of these trials should determine if autologous HSCT is better than current standards of care. Nonmyeloablative or reduced-intensity allogeneic transplantation protocols are being written, and advances in ex vivo stem cell expansion will soon be applied to autoimmune diseases to eliminate regimen-related neutropenia.

Historically, most autoimmune diseases are incurable, and it was impractical to define complete remission. HSCT, whether allogeneic or even autologous, may change this axiom. Initial results suggest that clinical tolerance, that is no evidence of disease off all immune-suppressive medications with normal third-party immune responsiveness, is being achieved in at least some patients. However, further improvement of the efficacy and safety of both autologous and allogeneic stem cell transplantation procedures need to be developed, and larger cohorts of patients need to be studied to assess the full benefits of stem cell transplantation as a most promising new armamentarium for the treatment of autoimmune diseases.

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bjh review

# Haemopoietic stem cell transplantation in autoimmune diseases: a European perspective

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## Summary

The potential of haemopoietic stem cell transplantation (HSCT) for the treatment of autoimmune and inflammatory diseases was originally supported by almost three decades of animal experiments and by the serindipitous remissions of autoimmune disease observed in patients undergoing transplantation for haematological disorders. Improved safety of both autologous and allogeneic HSCT over the last decade has been followed by increasing acceptance of HSCT as an experimental treatment for severe autoimmune diseases that are resistant to conventional treatment. International databases have collated over 700 procedures performed specifically for a variety of autoimmune diseases. Phase III clinical trials are in progress for some diseases. This review provides a comprehensive update on the efficacy and toxicity of HSCT in severe autoimmune disease. Future directions in the context of other evolving therapies are discussed.

Keywords: autoimmune diseases, haemopoietic stem cell transplantation, efficacy, toxicity.

The autoimmune diseases affect around 3–5% of the population. This heterogenous group of disorders, with features of autoimmunity, inflammation and defective repair processes, crosses many specialties. In organ-specific autoimmune diseases, such as autoimmune thyroiditis or insulin-dependent diabetes mellitus, the effects of target organ damage may be ameliorated by exogenous replacement therapy. Systemic diseases, such as scleroderma [systemic sclerosis (SSc)], systemic lupus erythematosis (SLE) and the systemic inflammatory arthritides, rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA), are less easy to treat. Although immunosuppression can provide disease control in some patients, such treatments are rarely, if ever, curative and may contribute substantially to long-term morbidity and mortality. In severely affected patients, the personal and societal costs of the autoimmune disorders and their treatments are high.

The last detailed appraisal of haemopoietic stem cell transplantation (HSCT) in autoimmune diseases in British Journal of Haematology was in 1997 (Snowden *et al*, 1997a). At that time, the field was on the cusp of translation from the laboratory to the clinic, a step facilitated by the improvements in safety associated with the use of cytokine-mobilized peripheral blood stem cells (PBSC) in the early 1990s. In 1997, only a small number of transplants were performed specifically for human autoimmune diseases and follow-up was short.

Over the last 7 years, substantial progress has been made. Information from animal experiments became translated into sporadic treatments and small phase I/II clinical trials. Analysis of larger series of patients reported to the European Blood and Marrow Transplant (EBMT) group/Autologous Blood and Marrow Transplant Registry (ABMTR) have expedited the evaluation of safety, outcomes and patient selection in specific disease groups. Promising results of single arm, phase I/II studies, have lead to several phase III randomized controlled trials. In addition, significant progress in non-myeloablative allogeneic transplantation occurring in parallel has made the clinical application of allogeneic transplantation in autoimmune disease more realistic. Increased understanding of stem cell plasticity has made possible the vision of regenerating tissues damaged by autoimmunity and inflammation. Laboratory assessment of immune reconstitution post-transplant is also becoming more sophisticated. Basic science and animal experiments continue to play an important role in our understanding of the mechanisms involved.

Notwithstanding, there has also been great progress in the understanding and treatment of autoimmune and inflammatory diseases outside the arena of transplantation. A prime example is the use of anti-tumour necrosis factor (TNF) drugs in RA and other inflammatory arthritides. Other autoimmune diseases, such as multiple sclerosis (MS) and scleroderma, however, have not witnessed breakthroughs of this magnitude

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and the lack of effective treatments continues to result in chronic intractable disease and disability.

## Rationale for the use of HSCT in autoimmune diseases

### *Animal studies*

Preclinical animal models, including spontaneous and induced forms, have implicated the lympho-haemopoietic system as pivotal in the pathogenesis of severe, refractory autoimmune disease and provided a platform for the clinical evaluation of HSCT in the treatment of these diseases in humans (van Bekkum, 1998, 2000). In the spontaneous or hereditary models, such as the murine BxSB lupus model and non-obese diabetic (NOD) mice, disease develops at high frequency with increasing age as a direct consequence of specific genetic defects. Alternatively, autoimmune disease can be induced in genetically susceptible strains by the immunization of specific antigens, including myelin basic protein, collagen and *Mycobacterium tuberculosis*. Induced models, such as the murine model of RA (adjuvant arthritis, AA) and the rat model of MS [experimental allergic encephalomyelitis (EAE)], are considered to more closely reflect the development of autoimmune diseases in humans (van Bekkum, 2000).

Initial studies demonstrated that spontaneous autoimmune diseases could be adoptively transferred by bone marrow transplantation (BMT) from affected into unaffected animals (Denman *et al*, 1969; Morton & Siegel, 1974; Ikehara *et al*, 1990). Further, Akizuki showed that T-cell depleted allografts could also transmit disease to the recipient (Akizuki *et al*, 1978). In contrast, induced autoimmune diseases were less readily transferred by transplantation, being predominantly determined by the recipient genetic susceptibility (van Bekkum, 1998). These data suggest that, while the haemopoietic stem cell (HSC) is of key importance in the pathogenesis of hereditary models of autoimmune disease (Ikehara *et al*, 1990), additional recipient variables, such as genetic susceptibility and environmental factors, may also be required for the transmission of inducible disease.

Subsequently, studies have shown that transplantation of normal allogeneic bone marrow, prevented and ameliorated or cured both spontaneous (Morton & Siegel, 1979; Ikehara *et al*, 1985a,b, 1989; Yasumizu *et al*, 1987; Adachi *et al*, 1995) and induced (van Bekkum *et al*, 1989; Kamiya *et al*, 1993; van Gelder & van Bekkum, 1995; Levite *et al*, 1995) autoimmune disease. Unexpectedly, a high incidence of, often durable, remission was also observed following autologous or pseudo-autologous BMT in induced AA (Knaan-Shanzer *et al*, 1991), EAE (van Gelder & van Bekkum, 1996) and myasthenia gravis (Pestronk *et al*, 1983). Further, the frequency of subsequent relapse could be reduced by transplants early in the course of disease (van Bekkum *et al*, 1989) and with the use of conditioning regimens of increasing intensity (van Bekkum, 2003). However, T-cell depletion of the autografts appeared to have no impact on the likelihood of relapse (van Bekkum, 2000).

The mechanisms of efficacy in the allogeneic setting is presumed to reflect a reduction in the burden of self-reactive lymphocytes by the conditioning regimen with eradication of residual immune cells by a postulated graft-*versus*-autoimmune (GVA) effect (Marmont, 2001) of the normal donor-derived immune system. The surprising efficacy of autologous BMT may result from a similar ablation of self-reactive lymphocytes during conditioning followed by induction of self-tolerance by re-education of HSC-derived lymphocytes. The published data on immune reconstitution after SCT are at present still limited and have not yet focused on the issue of tolerance induction.

Together, the animal studies of BMT in autoimmune disease show that these diseases may be adoptively transferred by allogeneic transplantation, allogeneic BMT may prevent and cure autoimmune disease and, unexpectedly, durable remissions may also be achieved following autologous BMT.

### *Anecdotal cases of adoptive transfer of autoimmune disease from donor to recipient*

The transfer of a broad range of autoimmune diseases from donor to recipient has now been documented (summarized in Table I), including diabetes mellitus, thyroiditis, psoriasis, myasthenia gravis, coeliac disease, colitis and immune thrombocytopenic purpura (ITP). It is unclear whether these diseases were transferred by the donor HSC, T cells or even other cells, such as mesenchymal stem cells (Moore *et al*, 2001). However, the transmission of thyrotoxicosis following infusion of $CD34^+$ selected human leucocyte antigen (HLA)-identical sibling bone marrow (Karthaus *et al*, 1997) demonstrates that substantial graft depletion of immunologically competent donor cells is insufficient to prevent the adoptive transfer of autoimmune disease.

The time interval from transplantation to the onset of clinical symptoms or signs in published cases has been highly variable, ranging from 5 months to 8 years (Table I). While this may reflect differences in the speed of evolution of diseases in different organs, it is likely that the immune suppression associated with graft-*versus*-host disease (GVHD) or its prevention and treatment would modify or delay the onset of overt autoimmune disease.

The aetiopathogenesis of autoimmune diseases in the context of allogeneic HSCT can be difficult to interpret for a number of reasons. First, the recipients are usually HLA-identical with their donors and as such may also have inherited a genetic susceptibility to autoimmune disease. Secondly, GVHD may mimic the clinical manifestations of many autoimmune disorders (Ferrara & Deeg, 1991; Sherer & Shoenfeld, 1998). Thirdly, endocrinopathies are well recognized complications of the chemoradiotherapy used in many conditioning regimens (Sanders, 1990; AlFiar *et al*, 1997). Finally, the immune dysregulation after HSCT may permit the

Table I. Anecdotal cases of adoptive transfer of autoimmune diseases.

| Autoimmune disease in donor | Indication for HSCT | Sex/age in years | Donor | Stem cell source | Manifestation of autoimmune disease in recipient | Reference |
|---|---|---|---|---|---|---|
| IDDM | AA | F/29 | HLA-matched brother | BM | Symptomatic IDDM + high titre ICA detected 4 years post-transplant | Lampeter *et al* (1993) |
| Thyroiditis | CML | M/41 | HLA-matched sister | BM | Thyrotoxicosis 9 months post-transplant with detectable antithyroid antimicrosomal antibodies | Aldouri *et al* (1990) |
| | AML | M/25 | HLA-matched sister | BM | Thyrotoxicosis 9 months post-transplant | Thomson *et al* (1995) |
| | – | M | HLA-matched sister | BM | Thyroid failure 4 years post-transplant with detectable antithyroid antimicrosomal antibodies | Wyatt *et al* (1990) |
| | AML | M/40 | HLA-matched sister | BM | Hyperthyroidism with antithyroglobulin antibodies 5 months post-transplant | Kishimoto *et al* (1997) |
| | AML | F/35 | HLA-matched sister | PBSC | Antithyroid antibodies 1 year post-transplant. Clinical hyperthyroidism after 2 years | Berisso *et al* (1999) |
| | AML | F/28 | HLA-matched sister | CD34 selected PBSC | Severe hyperthyroidism requiring plasmapharesis 13 months post-transplant | Karthaus *et al* (1997) |
| | AA | M/12 | HLA-matched sister | BM | Hyperthyroidism 8 years post-transplant with positive TSH-binding inhibitory antibodies | Holland *et al* (1991) |
| Polyendocrine failure | ALL | F | HLA-matched sister | BM | Symptomatic hypothyroidism and IDDM | Vialettes *et al* (1993) |
| ITP | AML | F/40 | HLA-matched brother | BM | Severe thrombocytopenia, which responded to IVIg. IgM anti-platelet antibodies documented in recipient and donor | Minchinton *et al* (1982); Waters *et al* (1983) |
| Psoriasis | CML | M/40 | Syngeneic | BM | Psoriasis developed 3 months post-transplant, which responded to topical steroids. 2 years after a second BMT from the same donor, extensive psoriasis was observed | Snowden & Heaton (1997) |
| | NHL | F/24 | HLA-matched brother | BM | Psoriasis developed at day 175 and responded within 3 weeks to topical coal tar | Gardembas-Pain *et al* (1990) |
| Myasthenia gravis | ALL | M/21 | – | BM | Myasthenia gravis 46 months post-transplant | Grau *et al* (1990) |
| Coeliac disease | AML | M/14 | HLA-matched sister | BM | Episodic diarrhoea begininning 4 months post-transplant with typical histology and anti-gliadin and anti-endomysium antibodies | Bargetzi *et al* (1997) |
| Ulcerative colitis | AML | M/37 | HLA-matched brother | BM | Ulcerative colitis 7 months post-transplant (brother had Crohn's disease) | Baron *et al* (1998) |
| Crohn's disease | HD | F/34 | MUD | PBSC | Ulcerative colitis 3·5 months post-transplant. Donor had NOD2/CARD15 mutation | Sonwalkar *et al* (2003) |

IDDM, insulin-dependent diabetes mellitus; AA, aplastic anaemia; AML acute myeloid leukaemia; CML, chronic myeloid leukaemia; NHL, non-Hodgkin's lymphoma; HD, Hodgkin's disease; ITP, immune thrombocytopenic purpura; BM, bone marrow; PBSC, peripheral blood stem cell; ICA, islet cell autoantibodies: TSH, thyroid-stimulating hormone; IVIg, intravenous immunoglobulin; BMT, bone marrow transplant; MUD, matched unrelated donor.

development of auto-immune phenomena (Lister *et al*, 1987; Rouquette-Gally *et al*, 1988; Drobyski *et al*, 1996; Chen *et al*, 1997).

However, the contention that these cases represent adoptive autoimmunity is supported by the observations that the recipient always developed the specific disease of the donor at


 

a time when lympho-haematopoiesis was fully donor-derived, and from the fact that the recipient had no clinical or serological evidence of disease prior to transplant (Table I). At present, the true incidence of this phenomenon is unknown as there has been no systematic evaluation and publication of specific cases is likely to have been influenced by reporting bias. It is notable that there has been no reported transfer of SLE or RA to date, despite transplantation of HSC from donors with these diseases (Sturfelt *et al*, 1996; Snowden *et al*, 1997b). It has been postulated that there may be fundamental differences between organ-specific and systemic autoimmune diseases which influence their ability to be transferred by transplantation (Snowden *et al*, 1997a). The recipient milieu may also promote the evolution of particular diseases by providing a favourable environment for the clonal expansion of donor-derived, tissue-specific, immunologically competent cells, perhaps by exposing organ-specific antigen as a consequence of the toxicity of the conditioning regimen.

### *Anecdotal case reports of cure of autoimmune disease in patients receiving autologous and allogeneic HSCT for other diseases*

The first indication that HSCT may ameliorate or even cure autoimmune disease came from anecdotal case reports of patients with a range of autoimmune diseases who were transplanted for coexistent malignancy or severe aplastic anaemia (Tables II and III). Although these cases were heterogeneous with respect to the type of conditioning regimen used, frequency of graft manipulation, method of GVHD prophylaxis and robustness of response evaluation, a number of important observations have emerged.

Autologous HSCT was associated with a rapid and complete remission of autoimmune disease in the majority of patients reported (Table II). However, these initial responses were often not sustained and early relapses were common (Euler *et al*, 1996; Cooley *et al*, 1997). This may reflect the persistence of autoreactive clones not eradicated by the conditioning regimen or infusion of autoreactive immunocompetent cells within the graft, or both. In keeping with the animal data, durable remissions (Kashyap & Forman, 1998) following autologous procedures have been documented and suggest that depletion of autoaggressive clones with subsequent re-education or 're-setting' of the HSC-derived immune system may be possible in some patients.

Allogeneic HSCT appeared to more often result in long-term remission of autoimmune disease (Table III), perhaps as a consequence of the infusion of a graft capable of normal immune reconstitution or the ability of the donor-derived immune system to exert a GVA effect. The existence of a GVA effect, analogous to graft *versus* malignancy seen in the context of allogeneic HSCT for leukaemia, is supported by the apparent superiority of allogeneic, compared with autologous, HSCT (Tables II and III), the resolution of autoimmune disease on withdrawal of ciclosporin (Slavin *et al*, 2000) or onset of GVHD (Slavin *et al*, 2000), the reappearance of disease with increasing recipient chimaerism (Lopez-Cubero *et al*, 1998), remission following non-myeloablative conditioning (Slavin *et al*, 2000) and the response of Crohn's disease in one patient to allogeneic, but not autologous HSCT (De Stefano *et al*, 1999).

However, allogeneic HSCT was not always associated with durable remission of autoimmune disease. Three patients with RA, psoriasis and Crohn's disease relapsed at 2, 1 and 1·5 years, respectively, despite lympho-haematopoiesis being fully donor-derived in at least two of them (McKendry *et al*, 1996; Snowden *et al*, 1998a,b,c). It is interesting that none of these patients had developed GVHD. Postulates of the mechanism permitting relapse of autoimmune disease despite fully donor-derived lymphopoiesis have included development of *de novo* disease because of the persistence of endogenous or exogenous 'triggers' in the context of genetic susceptibility, given that the donor was HLA identical to the recipient (Snowden *et al*, 1998a,b,c), persistence of recipient immune cells with 'education' of donor T cells (McKendry *et al*, 1996) or ongoing non-immune mediated tissue destruction (Tanaka *et al*, 1988; McKendry *et al*, 1996).

Despite the inherent reporting and publication bias of anecdotal cases, these reports offer proof of the principle that HSCT can cure or at least ameliorate autoimmune diseases in humans and have provided an impetus for the evaluation of this approach in prospective studies.

## HSCT in the specific treatment of autoimmune diseases

### *EBMT/EULAR and IBMTR/ABMTR autoimmune disease working groups*

In 1995, a proposal was published with a view to initiating interest in HSCT in autoimmune diseases (Marmont *et al*, 1995). The following year saw the first of several International Meetings in Stem Cell Therapy in Autoimmune Disease held in Basel under the auspices of the EBMT and the European League against Rheumatism (EULAR). Consensus guidelines were published and the International Autoimmune Disease Stem Cell Project Database was established. Similar developments progressed concurrently in North America and the International Bone Marrow Transplant Registry (IBMTR)/ ABMTR Working Group was formed. Further information is available on the appropriate websites (http://www.EBMT.org and http://www.ibmtr.org).

By 2004, the European and North American databases had accumulated almost 800 patients, the majority treated with autologous HSCT for a variety of indications (Table IV). A large number of phase I/II studies have now been published and prospective trials are ongoing. The remainder of this review aims to summarize the data, focussing on the larger disease groups.

Table II. Anecdotal case reports of cure/amelioration of autoimmune disease in patients receiving an autologous HSCT for other diseases.

| Autoimmune disease | Indication for HSCT | Sex/age in years | Stem cell source | Duration of remission | Time to relapse | Reference |
|---|---|---|---|---|---|---|
| RA | NHL | F/51 | PBSC | 5 weeks | 5 weeks | Euler *et al* (1996) |
| | NHL | M/54 | PBSC | 20 months | 20 months | Cooley *et al* (1997) |
| SLE | NHL | F/28 | PBSC | 352 d | 352 d | Euler *et al* (1996) |
| | CML | F/37 | BM | 30 months (maintenance 6-MP and MTX) | NR | Meloni *et al* (1997) |
| | NHL | F/56 | BM | 4 years | Antibodies detectable at 41 months | Snowden *et al* (1997c) |
| | HD | F/37 | PBSC | 34 months | NR | Schachna *et al* (1998) |
| Psoriasis | NHL | M/35 | PBSC | 22 months | 22 months | Cooley *et al* (1997) |
| | APL | M/54 | BM | 14 months | NR | Cooley *et al* (1997) |
| | Plasma cell leukaemia | F/40 | PBSC | 8 months | 8 months | Cooley *et al* (1997) |
| Myasthenia gravis | Ovarian cancer | F/52 | PBSC × 2 | 6 months | 6 months | Euler *et al* (1996) |
| | NHL | M/38 | CD34 selected PBSC + BM | 250 d | NR | Salzman (1994) |
| MS | Ph + AL | M/35 | PBSC | 1 month | NR | Meloni *et al* (1999) |
| | ALL | F/43 | BM | Stabilization for 6 months | – | Mandalfino *et al* (2000) |
| | NHL | F/47 | BM | Stabilization for 40 months | – | Mandalfino *et al* (2000) |
| Ulcerative colitis | Breast cancer | F | PBSC | 2 years | NR | Castro *et al* (1996) |
| | Breast cancer | F/57 | PBSC | 36 months | NR | Marti *et al* (2001) |
| Crohn's disease | Breast cancer | F | PBSC | 2 years | NR | Castro *et al* (1996) |
| | HD | M/28 | PBSC | 1155 d | NR | Musso *et al* (2000) |
| | NHL | M/20 | BM | 7 years | NR | Kashyap & Forman (1998) |
| | AML | F/54 | PBSC | 5 years | NR | Soderholm *et al* (2002) |
| ITP | Lung cancer | M/46 | PBSC | 180 d | NR | Demirer *et al* (1999) |
| AIHA | CLL | M/48 | CD34 selected PBSC | 23 months | 23 months (simultaneous relapse of CLL) | Jindra *et al* (1999) |
| Ankylosing spondylitis | NHL | M/57 | PBSC | 20 months | NR | Jantunen *et al* (2000) |
| Sjögren's syndrome | NHL | F/35 | PBSC | 2 months | Remained steroid dependent | Rosler *et al* (1998) |
| | NHL | F/42 | Not stated | No remission | – | Ferraccioli *et al* (2001) |

RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; MS, multiple sclerosis; AML acute myeloid leukaemia; CML, chronic myeloid leukaemia; APL, acute promyelocytic leukaemia; ALL acute lymphoblastic leukaemia; CLL, chronic lymphocytic leukaemia; NHL, non-Hodgkin's lymphoma; HD, Hodgkin's disease; BM, bone marrow; PBSC, peripheral blood stem cell. 6 MP, mercaptopurine; MTX, methotrexate; NR, not recorded.

### *Multiple sclerosis*

Multiple sclerosis is an incurable, physically and psychologically debilitating disease thought to be mediated by both an immune-mediated inflammatory loss of myelin and a degenerative loss of axons (Compston & Coles, 2002). The clinical course is very heterogeneous; the presentation in 80% of patients is initially relapsing and remitting with the remainder having progressive deterioration from the onset (primary progressive) (Compston & Coles, 2002). Within 10 years, approximately one-half of patients with relapsing and remitting disease will develop progressive features (secondary progressive). Conventional immunosuppressive and immunomodulatory therapies such as α-interferon, copaxone, pulsed intravenous steroids, cyclophosphamide and mitoxantrone have unsatisfactory efficacy in controlling progressive disease and long-term disability. Historical, longitudinal data suggests that half of the patients will loose the ability to walk unaided within 15 years of diagnosis and overall life expectancy is reduced by around 10 years (Weinshenker *et al*, 1989; Cottrell *et al*, 1999).

Initial interest in the therapeutic potential of HSCT for MS was prompted in 1995 (Burt *et al*, 1995) by the efficacy of this

Table III. Anecdotal case reports of cure/amelioration of autoimmune disease in patients receiving an allogeneic HSCT for other diseases.

| Autoimmune disease | Indication for HSCT | Recipient sex/age in years | Donor | Stem cell source | GVHD | Duration of remission | Time to relapse | Reference |
|---|---|---|---|---|---|---|---|---|
| RA | SAA | F/28 | HLA-matched brother | BM | Acute and chronic | 13 years | NR | Lowenthal *et al* (1993), Snowden *et al* (1998c) |
| | SAA | F/34 | HLA-matched brother | BM | Acute and chronic | 14 years | Relapsed at 2 and 3 years. No treatment for 11 years | Lowenthal *et al* (1993), Snowden *et al* (1998c) |
| | SAA | F/53 | HLA-matched brother | BM | None | 2 years | 2 years | McKendry *et al* (1996) |
| | SAA | F/33 | HLA-matched sibling | BM | Chronic | 9 years | NR | Jacobs *et al* (1986), Snowden *et al* (1998c) |
| | SAA | Four patients | HLA-matched sibling | BM | Acute in 1 | 2 years in 1 | NR in 1 | Baldwin *et al* (1977) |
| SLE | SAA | F/30 | HLA-matched brother | BM | Acute | 15 years | NR | Gur-Lavi (1999) |
| MS | CML | F/46 | HLA-matched brother | BM | None | 14 months | NR | McAllister *et al* (1997) |
| | AML | M/43 | HLA-matched sibling | BM | Grade I acute GVHD skin | 48 months | NR | Mandalfino *et al* (2000) |
| Autoimmune hepatitis | ALL | M/19 | HLA-matched brother | TCD-BM + DLI day 1 | Grade I acute GVHD skin | 51 months | NR | Vento *et al* (1996) |
| Psoriasis | AML | F/40 | HLA-matched sister | BM | Chronic GVHD buccal mucosa | 24 months | NR | Kishimoto *et al* (1997) |
| | – | – | – | BM | None | 4 years | NR | Yin & Jowitt (1992) |
| | AML | – | – | BM | None | 5 years | NR | Eedy *et al* (1990) |
| | CML | M/38 | HLA-matched sister | PBSC | Acute and chronic | 3 years | NR | Slavin *et al* (2000) |
| | CML | M/56 | HLA-matched sister | BM | None | 1 year | 1 year | Snowden *et al* (1998c) |
| Ulcerative colitis | AML | – | – | BM | None | 4 years | NR | Yin & Jowitt (1992) |
| Crohn's disease | CML in 5; AML in 1 | Six patients | Five sibling, 1 URD | BM | Acute in 5; chronic in 4 | 4·5–15·3 years in 5/6 | 1·5 years in 1/6 | Lopez-Cubero *et al* (1998) |
| Shulman's syndrome | SAA | M/43 | HLA-matched sister | BM | Chronic GVHD of skin and liver | 34 months | NR | Cetkovsky *et al* (1998) |
| AIHA | Thalassaemia intermedia | M/12 | URD | BM | None | 18 months | NR | De Stefano *et al* (1999) |
| Alopecia | CML | M/40 | HLA-matched sister | PBSC | – | 2 years | NR | Seifert *et al* (2004) |

RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; MS, multiple sclerosis; AIHA, auto immune haemolytic anaemia; AML acute myeloid leukaemia; CML, chronic myeloid leukaemia; ALL acute lymphoblastic leukaemia; SAA, severe aplastic anaemia; URD, unrelated donor; BM, bone marrow; PBSC, peripheral blood stem cell; NR, not recorded.

Table IV. Current status of HSCT for autoimmune diseases.

| | EBMT | IBMTR/ ABMTR* |
|---|---|---|
| Transplant type | | |
| Autologous | 521† | 186 |
| Allogeneic | 26 | 23 |
| Indication | | |
| Neurological | | |
| Multiple sclerosis | 161 | 77 |
| Amyotrophic lateral sclerosis | 2 | 4 |
| Myasthenia gravis | 2 | 1 |
| Guillain–Barre syndrome | 1 | |
| Other | 3 | 1 |
| Rheumatological | | |
| Systemic sclerosis | 88 | 32 |
| Systemic lupus erythematosis | 66 | 55 |
| Rheumatoid arthritis | 72 | 6 |
| Juvenile idiopathic arthritis | 54 | 2 |
| Dermatomyositis | 7 | |
| Wegener's granulomatosis | 6 | |
| Bechet's syndrome | 5 | 1 |
| Mixed connective tissue disease | 4 | |
| Cryoglobulinaemia | 4 | |
| Psoriatic arthritis | 2 | |
| Ankylosing spondylitis | 2 | |
| Vasculitis | 2 | 1 |
| Sjögren's/sicca syndrome | 1 | 1 |
| Polychondritis | 1 | |
| Other | 1 | 3 |
| Haematological | | |
| Idiopathic thrombocytopenic purpura | 12 | 6 |
| Autoimmune haemolytic anaemia | 5 | |
| Pure red cell aplasia | 4 | |
| Thrombotic thrombocytopenic purpura | 3 | |
| Evan's syndrome | 2 | 2 |
| Pure white cell aplasia | 1 | |
| Other | | 2 |
| Others | | |
| Inflammatory bowel disease | 5 | 10 |
| Others | 6 | 5 |

*The data presented here are preliminary and were obtained from the Statistical Center of the International Bone Marrow Transplant Registry (IBMTR) and Autologous Blood and Marrow Transplant Registry (ABMTR). The analysis has not been reviewed or approved by the Advisory or Scientific Committees of the IBMTR and ABMTR.
†521 reports, four double transplants, 517 mobilized, 509 transplanted.

approach following autologous or pseudoautologous HSCT in EAE animals and anecdotal reports of neurological response following autologous and allogeneic HSCT for concurrent haematological malignancies as discussed above (Tables II and III).

To date over 200 patients have undergone autologous HSCT specifically for MS (Table V). The largest series, including 85 patients from 20 centres, has been reported by the EBMT registry (Fassas *et al*, 2002). The median age of the patients was 39 years (range 20–58 years), there was a female preponderance (61%) and the median time interval from diagnosis to transplant was 7 years (range 1–29 years). Almost all patients had progressive MS (26% primary, 70% secondary) with a median Expanded Disability Status Scale (EDSS) of 6·5 (range 4·5–8·5) at the time of transplant. PBSC were most frequently mobilized with cyclophosphamide 1·5–4 g/m$^2$ and granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage (GM)-CSF (86%) or with G-CSF alone (11%). An adequate HSC dose was achieved in all patients although two required a second procedure. Four patients (6%) experienced a neurological improvement of one EDSS point or greater following mobilization. However, transient neurological deterioration associated with priming was also observed in three individuals; haemiparesis in one, leg paresis in one and seizures in one patient. The conditioning regimens used were highly variable, with BEAM [BCNU (carmustine), etoposide, cytarabine, melphalan] + anti-thymocyte globulin (ATG) being the most frequent (47%). Ninety-three percent of patients received PBSC and 7% BM, of which 61% were CD34$^+$ selected.

Autologous HSCT in these patients was associated with significant toxicity. Twenty-two of the 83 evaluable cases (27%) experienced neurological deterioration during the transplant procedure, which was permanent in six (7%) and fatal in two patients (2%). A further five (6%) patients died of cardiac toxicity in one, cerebral aspergillosis in one, septicaemia in one, influenza pneumonitis in one and one episode of postoperative pneumococcal septicaemia 19 months post-transplant. All these five patients had received CD34$^+$ selected grafts. Fig 1 demonstrates overall survival for these patients.

Eighteen (21%) patients improved by an EDSS score of 1 or greater following transplantation, although six of these subsequently progressed. Progression-free survival (PFS) at 3 years was 74 ± 12% (Fig 2), with a 3-year risk of progression of disability of 20%. While older age was significantly associated with an inferior PFS there was also a non-significant trend towards an inferior PFS in those with primary progressive disease and those with a longer duration from diagnosis to transplant. All patients with magnetic resonance imaging (MRI) evidence of active CNS inflammation prior to transplant (22 patients) had complete radiological resolution of these signs post-transplant. Follow-up of the study was short (median 16 months, range 3–59), thereby precluding any long-term evaluation of efficacy. However, the 3-year PFS of 74% in these high-risk patients indicates a possible benefit of this approach, although this was gained at the cost of substantial toxicity.

Other smaller studies, some of whose patients are included in the EBMT analysis, have also suggested that durable stabilization of MS can be achieved by autologous HSCT (Table V). Those most likely to benefit from the procedure appear to be younger (Fassas *et al*, 2002), have an EDSS score of <6 at the time of transplant (Burt *et al*, 2003a), have relapsing–remitting or secondary progressive disease (Nash *et al*, 2003) and are transplanted at a shorter time interval from

Table V. Phase I/II studies evaluating autologous HSCT for multiple sclerosis.

| Institution | No. of patients | Mobilization regimen | Conditioning regimen | Graft manipulation | Unexpected toxicity | Response | Time to relapse | Reference |
|---|---|---|---|---|---|---|---|---|
| EULAR/ EBMT/ IBMTR | 85 | BM (6); PBSC (79) | Various | Various | 6% TRM | No progression in 72%, improvement in 21% | – | Fassas *et al* (2002) |
| Chicago | 21 | G-CSF alone (4) Cy 2 g/m$^2$ + G-CSF (17) | Cy 120 mg/kg + TBI + MP | Positive CD34$^+$ selection | Engraftment syndrome in 5/21 | No progression in 9/9 patients with EDSS ≤6·0; progression in 8/12 with EDSS > 6·0 | – | Burt *et al* (1998, 2003a) |
| Seattle, Colorado, Nebraska, City of Hope, Duke University, Washington, Texas | 26 | G-CSF | Cy 120 mg/kg, TBI + ATG | Positive CD34$^+$ selection | Engraftment syndrome in 13/18 | 27% risk of progression at 3 years | – | Nash *et al* (2003) |
| Thessaloniki | 24 | Cy 4 g/m$^2$ + G-CSF | BEAM + ATG | Positive CD34$^+$ selection (9), none (15) | None | 3-year PFS was 92% for secondary progressive disease and 39% for primary progressive | 9/18 initial responders relapsed | Fassas *et al* (1997, 2000) |
| Barcelona | 15 | Cy 3 g/m$^2$ + G-CSF | BCNU 300 mg/m2 + Cy 150 mg/kg + ATG | Positive CD34$^+$ selection | Engraftment syndrome in 3/15 | Disease stabilized or improved in 12/15 | 2/12 initial responders relapsed | Saiz *et al* (2001), Carreras *et al* (2003) |
| GITMO-NEURO intergroup | 10 | Cy 4 g/m$^2$ + G-CSF | BEAM | None | None | No progression in 10/10 at median of 15 months | – | Mancardi *et al* (2001) |
| Prague | 10 | Cy 4 g/m$^2$ + G-CSF | BEAM ± ATG (3) | T-cell depletion (7) | None | No progression in 9/10 at median of 9 months | – | Kozak *et al* (2000, 2001) |
| City of Hope | 5 | G-CSF | Bu 16 mg, Cy 120 mg/kg + ATG | Positive CD34$^+$ selection | None | No progression in 3/4 at 18–30 months | – | Openshaw *et al* (2000) |
| Nebraska/Seattle | 3 | G-CSF | Cy 120 mg/kg + TBI + ATG | None | None | Disease stable at 2 years in 1/3 | – | McGuire *et al* (2003) |

Data from many of the patients reported in the smaller studies were included in the EULAR/EBMT/IBMTR report (Fassas *et al*, 2002).

BM, bone marrow; PBSC, periphral blood stem cells; G-CSF, granulocyte colony-stimulating factor; Cy, cyclophosphamide; TBI, total body irradiation; MP, methyl prednisolone; ATG, anti-thymocyte globulin; BCNU, carmustine; BEAM, BCNU, etoposide, cytarabine, melphalan; TRM, treatment-related mortality; EDSS, Expanded Disability Status Scale; PFS, progression-free survival.

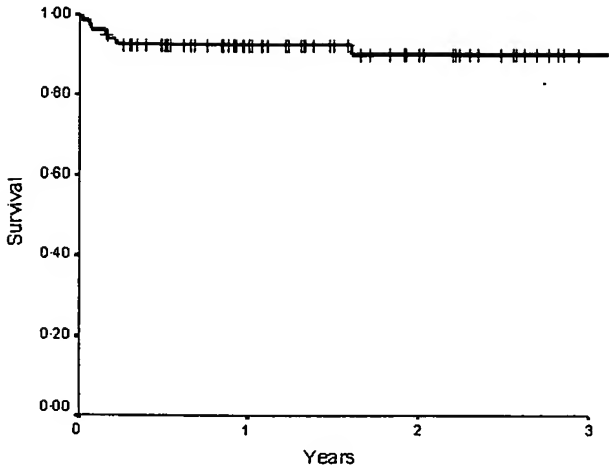


Fig 1. Overall survival following autologous HSCT For MS. From: Fassas *et al* (2002). Reproduced by permission of Steinkopff Verlag, Germany.

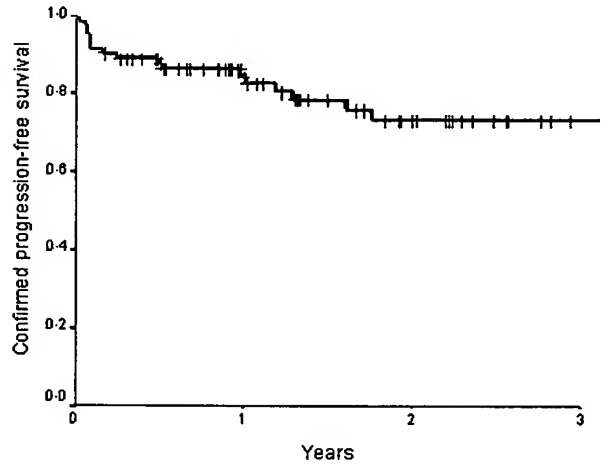


Fig 2. Progression-free survival following autologous HSCT for MS. From Fassas *et al* (2002). Reproduced by permission of Steinkopff Verlag, Germany.

diagnosis (Fassas *et al*, 2002). The immediate and sustained anti-inflammatory effect of the conditioning regimen can be readily demonstrated by MRI and appears to be superior to that achieved with other immunosuppressive therapies (Burt *et al*, 2001; Mancardi *et al*, 2001; Saiz *et al*, 2001; Kimiskidis *et al*, 2002; Fassas & Kimiskidis, 2003). However, oligoclonal bands may still persist in the cerebrospinal fluid for up to 2 years post-transplant (Openshaw *et al*, 2000; Saiz *et al*, 2001; Nash *et al*, 2003) and neurodegenerative axonal atrophy appears to continue even when active inflammatory demyelination has been switched off (Burt *et al*, 2001; Saiz *et al*, 2001; Kimiskidis *et al*, 2002).

The optimal conditioning regimen for patients with MS remains an unresolved issue. A variety of regimens have been used thus far, predominantly using either BEAM or cyclophosphamide and total body irradiation (TBI), with or without additional ATG. The numbers of patients treated with each regimen are too small to draw any comparisons in terms of efficacy or safety. However, the documentation of infectious deaths mandate caution in the combined use of T-cell-depleted grafts with highly immunosuppressive conditioning regimens. Non-TBI containing regimens would also seem to be preferable given the known long-term complications associated with its use, the theoretical risk of exacerbating axonal loss via generation of free radicals and animal experiments in which neurological deterioration was associated with the use of TBI (van Gelder & van Bekkum, 1995).

Despite the theoretical advantage of depleting the graft of autoreactive T cells, the value of graft manipulation is also unclear. Although CD34-selection has not been shown to influence PFS (Fassas *et al*, 2002) 32 of the 33 patients receiving an unmanipulated graft in this study had also received ATG as part of the conditioning regimen which would have mediated lymphocyte reduction and potentially confounded this observation. However, it is clear that a substantial risk of serious infection has been documented in patients who have received manipulated grafts (Fassas *et al*, 2000; Openshaw *et al*, 2000; Fassas & Kimiskidis, 2003).

An engraftment syndrome constituting fever, rash, fatigue and neurological deterioration may occur in up to 26% patients undergoing autologous HSCT for MS (Oyama *et al*, 2002). Although, generally self-limiting, the syndrome may require intervention with corticosteroids. Whether there is a role for prophylactic steroid therapy around the time of engraftment will need to be determined in the context of a randomized trial.

At present, follow-up is too short to determine the risk of late complications arising from the procedure. Of note is the occurrence of autoimmune thyroiditis 11 months post-transplant in one patient and a refractory, acquired haemophilia A which resulted in death 28 months from transplantation in another (Fassas *et al*, 2002). A further patient who received a CD34-selected graft and rabbit ATG in the conditioning regimen has also died from Epstein–Barr virus (EBV)-associated post-transplant lymphoproliferative disorder (Nash *et al*, 2000).

The principal aim of therapy for MS is to arrest neurological deterioration and prevent disability. The worldwide experience to date suggests that autologous HSCT has a substantial anti-inflammatory effect with improvement in MRI lesions and an apparent early stabilization of disease. It remains essential to determine that quality of life is improved by the procedure and meticulous patient selection will be crucial in optimizing outcome. The contribution of degenerative disease to neurological deterioration must also be defined, as this is unlikely to be influenced by HSCT. The extent to which HSC-derived cells could replace damaged neurones (Mezey *et al*, 2000, 2003; Kim *et al*, 2002a) is a theoretical, but exciting prospect.

At present, autologous HSCT remains an experimental treatment option for MS and the relative efficacy and toxicity of this approach compared with conventional therapy must be


 

formally evaluated in the context of a prospective, randomized study. An EBMT/EULAR initiative, the Autologous Stem Cell Transplantation International Multiple Sclerosis Trial (ASTIMS; http://www.euro-meds.com/download/astims_trial.pdf) study is now recruiting patients and will compare autologous HSCT (using BEAM and ATG and unselected cyclophosphamide and G-CSF-mobilized PBSC) with the best standard care and Mitoxantrone 20 mg per month for 6 months. The inclusion criteria are (i) age between 18 and 50 years; (ii) established diagnosis of MS according to clinical and MRI criteria, as defined by Poser *et al* (1983); (iii) EDSS between 3·5 and 6·5 at screening evaluation; (iv) secondary progressive MS with or without relapses with increasing EDSS and progressive MRI lesions in spite of immunomodulating therapy; (v) relapsing–remitting MS with increasing disability and increasing EDSS and progressive MRI lesions in spite of immunomodulating therapy.

Well-designed clinical trials, such as this, will be crucial in defining the appropriate place of autologous HSCT in the therapeutic armamentarium of MS.

### *Systemic sclerosis*

Systemic sclerosis (SSc) is a rare, multi-system disorder characterized by skin and visceral (lung, cardiac, gastrointestinal and renal) fibrosis as a consequence of excessive collagen deposition. Patients with the diffuse variant, who have extensive skin and early visceral involvement, have a poor outcome with a 5-year mortality rate of around 50% (Medsger *et al*, 1971; Altman *et al*, 1991; Nagy & Czirjak, 1997; Bryan *et al*, 1999). The aetiopathogeneis of SSc is poorly understood, but an immune element is suggested by the presence of autoantibodies (Harvey & McHugh, 1999), its association with other autoimmune disorders (Burt *et al*, 2003b), the clinical and pathological similarities of this disease with chronic GVHD (Bos *et al*, 1988) and the response of patients with SSc-associated alveolitis to cyclophosphamide (White *et al*, 2000). Given its natural history and the current lack of effective treatment, SSc has been an obvious candidate for evaluation of experimental therapies such as autologous HSCT.

Phase I/II studies, case reports and early registry data (Table VI) have suggested that autologous HSCT is associated with a subjective and objective improvement in skin flexibility in the majority of patients (Tyndall *et al*, 1997; van den Hoogen *et al*, 1999; Martini *et al*, 1999; Binks *et al*, 2001; Farge *et al*, 2002; McSweeney *et al*, 2002; Burt *et al*, 2003b) and a probable stabilization of pulmonary involvement (Tyndall *et al*, 1997; Martini *et al*, 1999; Binks *et al*, 2001; McSweeney *et al*, 2002). However, high mortality rates (27%) associated with priming regimens and the transplant procedure were reported, because of both disease progression (10%) or transplant-related complications (17%) (Binks *et al*, 2001). Notably, one patient died of cardiac fibrosis 2 d after transplant, despite normal, detailed pretransplant cardiac assessment (Rosen *et al*, 2001) and highlights the specific challenge of developing safe transplantation regimens in patients with multisystem connective tissue disorders.

Farge *et al* (2004) have recently updated the earlier report of Binks *et al* (2001) summarizing the European experience of autologous HSCT in SSc. Fifty-seven patients with severe SS from 22 centres in nine countries were registered in the EBMT/EULAR International Stem Cell Research project database up to August 2002. The median age was 40 years (range 9·1–68·7 years), the male to female ratio was 10:47 and the median duration of disease prior to transplant was 36 months (range 2·2–159·4). PBSC mobilization using G-CSF with or without cyclophosphamide was successful in all but one patient, in whom an adequate BM harvest was obtained. *Ex vivo* T-cell depletion of the graft was performed in 87% of cases, most commonly by CD34 selection (69%). The conditioning regimen used varied between centres but engraftment was achieved in all patients.

Strategies to improve outcome by more stringent patient selection criteria based on learned experience was reflected in a lower treatment-related mortality (TRM; 8·7%) than previously reported. Death related to disease progression occurred in 14% and the overall survival at 5 years was 72%. Overall, a partial or complete response was observed in 92% of the 50 patients who had been followed up for at least 6 months post-transplant. A significant reduction in the median skin scores was observed up to 36 months post-transplant (Fig 3) and 79% of patients with 2 years of follow-up had maintained a reduction in skin scores of greater than 25% compared with baseline. Renal and pulmonary involvement did not change significantly after transplant, possibly reflecting stabilization of prior irreversible organ damage. Thirty-five percent of initial responders subsequently relapsed within a median of 10 months (range 2·2–48·7 months), although not to pre-transplant activity, and the 5-year cumulative probability of relapse was 48%. These data confirm the feasibility and increasing safety of autologous HSCT for SSc. Current transplant strategies do not appear to be curative, but do offer a clinically meaningful delay in disease progression.

The EBMT and EULAR are now conducting a prospective randomized study, the Autologous Stem Cell Transplantation International Scleroderma trial, in which autologous HSCT (using 200 mg/kg cyclophosphamide and 7·5 mg/kg ATG and CD34-selected autologous cyclophosphamide and G-CSF mobilized PBSC) will be compared with 12 monthly doses of 750 mg/m$^2$ cyclophosphamide. The eligibility criteria for entry into the study include (i) age between 16 and 60 years; (ii) established diagnosis of systemic sclerosis according to American College of Rheumatology (ACR) criteria (Felson *et al*, 1995) and (iii) diffuse scleroderma with disease duration ≤4 years as development of first sign of skin thickening *plus* modified Rodnan skin score ≥15 *plus* major respiratory, renal or cardiac involvement (with documented evidence of onset or clinically significant worsening in the previous 6 months). This exciting study will be crucial in defining the role of autologous HSCT in the challenging management of SSc.

Table VI. Phase I/II studies evaluating autologous HSCT for systemic sclerosis.

| Institution | No. of patients | Mobilization regimen | Conditioning regimen | Graft manipulation | Unexpected toxicity | Response | Time to relapse | Reference |
|---|---|---|---|---|---|---|---|---|
| EULAR/EBMT | 57 | PBSC (55); BM (2) | Various | Positive $CD34^+$ selection (48) | 8·7% TRM | PR 13/32 + CR14/32 at 20 months | 35% relapsed at 2·2–48·7 months | Farge *et al* (2004) |
| EULAR/EBMT | 41 | Various | Various | Positive $CD34^+$ selection (38) | 17% TRM | 69% | 19% after a median of 67 d | Binks *et al* (2001) |
| Seattle | 19 | G-CSF | Cy 120 mg/kg, + ATG + TBI | Positive $CD34^+$ selection | 3/19 TRM | 12/12 at 1 year | – | McSweeney *et al* (2002) |
| ISAMAIR | 12 | Cy 4 g/m$^2$ + G-CSF | Cy 200 mg/kg or Mel 140 mg/m$^2$ | Positive $CD34^+$ selection | 1/11 TRM | 8/11 at 18 months | 5/8 relapsed at 9–12 months | Farge *et al* (2002) |
| Nijemegen | 5 | Cy 4 g/m$^2$ + G-CSF | Cy 200 mg/kg | Positive $CD34^+$ selection | None | 45% improvement in skin score at 2–16 months | – | van den Hoogen *et al* (1999) |
| Berlin | 1 | Cy 2 g/m$^2$ | Cy 200 mg/kg + ATG + MP | – | Death day +2 due to advanced cardiopulmonary fibrosis | – | – | Rosen *et al* (2001) |
| Basel | 1 | Cy 2 g/m$^2$ + G-CSF | Cy 200 mg/kg | Positive $CD34^+$ selection, negative CD3 selection | None | 1/1 | None at 6 months | Tyndall *et al* (1997) |
| Pavia | 1 | Cy 4 g/m$^2$ + G-CSF | Cy 200 mg/kg + CAMPATH | Positive $CD34^+$ selection | None | 1/1 | None at 2 years | Martini *et al* (1999) |

Data from many of the patients reported in the smaller studies were included in the EULAR/EBMT report (Farge *et al*, 2004), which represents an update of the results published by Binks *et al* (2001). ISAMAIR, Intensification et Autogreffe dans les Maladies Auto Immunes Resistantes Study Group; BM, bone marrow; PBSC, periphral blood stem cells; G-CSF, granulocyte colony-stimulating factor; Cy, cyclophosphamide; Mel, melphalan; MP, methyl prednisolone; TRM, treatment-related mortality; PR, partial response; CR, complete remission.

 

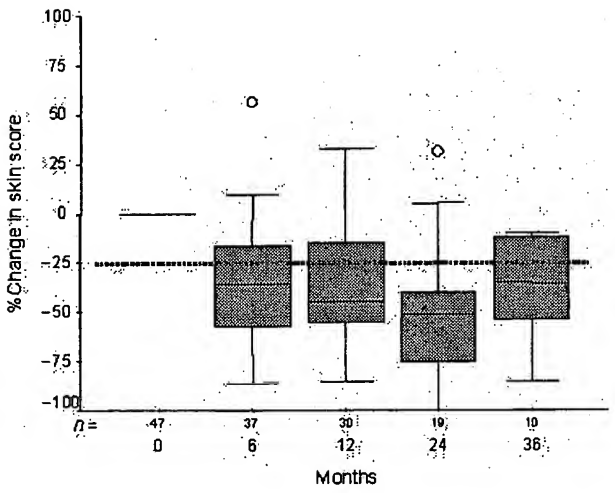


Fig 3. Response in median skin score following autologous HSCT For Ssc. From: Farge *et al* (2004). Reproduced with permission from the BMJ Publishing Group.

### *Rheumatoid arthritis*

Rheumatoid arthritis affects approximately 1% of the population and is a significant source of morbidity and, in the long-term, mortality. A proportion of RA patients respond poorly and/or only transiently to disease modifying anti-rheumatic agents (DMARDs) and progress inexorably to severe disability. It is in this resistant group of patients that HSCT has been considered most appropriate.

Most RA patients can be mobilized to produce sufficient progenitors for autologous transplantation despite prior exposure to multiple and prolonged exposure to myelotoxic agents (McGonagle *et al*, 1997; Snowden *et al*, 1998a; Breban *et al*, 1999; Snowden *et al*, 1998b). Flare may be seen on exposure to G-CSF alone (Snowden *et al*, 1998b), but appears to be rare when mobilization chemotherapy is used (Breban *et al*, 1999). In some patients, remissions lasting up to 6 months may be induced by the priming regimen (Breban *et al*, 1999).

The first procedure specifically for RA was performed in Perth in 1996 (Joske *et al*, 1997). A wheelchair-dependent patient previously treated with over 10, second line therapies was mobilized with cyclophosphamide 4 g/m$^2$ and G-CSF followed by cyclophosphamide 200 mg/kg and an unmanipulated autologous PBSC rescue. The patient maintained an ACR response of 70 for 25 months. Following an increase in disease activity, re-introduction of methotrexate 10 mg weekly (which had previously been unsuccessful) controlled the disease for a further 12 months to last reported follow-up (Snowden *et al*, 2004a).

Since this first report, an accumulating body of evidence from case series and studies has established the relative safety of autologous HSCT in severe RA (Table VII). The largest of these has been the recent composite report based on the EBMT/ABMTR registries data of 73 patients (Snowden *et al*, 2004b). Patients, who were predominantly female and rheumatoid factor positive, had been previously treated with an average of 5 (range 2–9) DMARDs and were significantly disabled. The majority of patients received high dose cyclophosphamide-based regimens with or without ATG. Almost all patients had clinically significant initial responses to the autograft alone (Fig 4), but by 6–12 months most patients were re-started on DMARDs for persistent or recurrent disease activity. Interestingly, there were clinically meaningful, with some profound, responses to DMARD re-introduction in about half of these patients, including agents to which patients had previously been deemed resistant.

Some durable responses are seen with transplant alone, especially in seronegative RA. There is also a suggestion that increasing dose intensity might produce more favourable results although increased toxicity may restrict this approach. For example, with the use of the busulphan/cyclophosphamide regimen and highly purified stem cell rescue, one patient with seronegative RA remains in remission at 56 months with complete reconstitution of the T-cell repertoire to pre-transplant levels. However, the one other patient treated with this myeloablative protocol also achieved complete remission by 3 months, but died of lung carcinoma and infection at 5 months (Snowden *et al*, 2004a). Although probably unrelated to the transplant procedure, this case is the only post-transplant death documented in RA, and caution must be advised with respect to dose intensification.

Many studies have used T-cell depleted or other forms of graft manipulation (Table VII), largely based on laboratory studies and hypothetical risk of re-infusion of pathogenic T cells within the graft. However, prospective and retrospective data has provided no support for graft manipulation strategies as a means of improving outcome (Moore *et al*, 2002; Snowden *et al*, 2004b). Whether graft manipulation would have a greater impact in the context of a lower autoreactive T-cell burden following more intensive conditioning regimens remains unknown, but it is likely that such an approach would be associated with prohibitive toxicity.

Viewed in isolation, the effect of the high-dose cyclophosphamide-containing regimens with autologous transplant alone is possibly disappointing in the majority of rheumatoid factor positive patients. However, if appraised as the product of both transplant plus the post-transplant salvage and maintenance DMARD therapy, overall responses, including highly significant reduction in the level of disability, can be considered impressive, at least in the medium term, in this group of patients with resistant and aggressive disease. In addition, such responses are achieved with relative safety.

The lack of sustained responses in many patients has led some investigators to explore syngeneic and allogeneic HSCT, with the hope that RA might be cured by a 'one-hit' treatment. The Royal Melbourne Hospital reported a successful syngeneic transplant in severe seronegative RA, with complete remission of disease at over 5 years of follow-up (McColl *et al*, 1999; Snowden *et al*, 2004a). One reduced intensity allogeneic

Table VII. Phase I/II studies evaluating autologous HSCT for severe rheumatoid arthritis.

| Institution | No. of patients | Mobilization regimen | Conditioning regimen | Graft manipulation | Unexpected toxicity | Response | Time to relapse | Reference |
|---|---|---|---|---|---|---|---|---|
| EBMT/ABMTR | 76 | PBSC (72); BM (1) | Cy 200 mg/kg (62), various | T depletion (45); Unmanipulated (28) | 1 death due to lung cancer + infection at 5 months | 67% at least ACR50% | Most restarted DMARDs within 6 months | Snowden *et al* (2004b), Tyndall *et al* (2001), Tyndall *et al* (1999) |
| Sydney/ | 18 | G-CSF | Cy 200 mg/kg | Positive $CD34^+$ selection | None | 70% no difference between groups | Median 147 d | Moore *et al* (2002) |
| Queensland/ Perth/Brisbane/ Melbourne | 15 | G-CSF | Cy 200 mg/kg | None | | | Median 180 d 4/33 maintained remission at 11–17 months | |
| Leiden/Utrecht/ Nijmegan | 14 | Cy 4 g/$m^2$ + G-CSF | Cy 200 mg/kg | Positive $CD34^+$ selection | None | 7/10 | – | Verburg *et al* (2001) |
| Sydney | 4 | G-CSF | Cy 100 mg/kg | None | None | 4/4 | 3–4 months in 4/4 | Snowden *et al* (1999) |
| | 4 | G-CSF | Cy 200 mg/kg | None | None | 4/4 | 3–19 months in 3/4 | |
| Leeds | 6 | Cy 2 g/$m^2$ + G-CSF | Cy 200 mg/kg | Positive $CD34^+$ selection, negative CD4/8 cell selection | None | 6/6 | 1·5–9 months in 6/6 | Bingham *et al* (2001b) |
| Chicago/ Wisconsin | 4 | Cy 2 g/$m^2$ + G-CSF | Cy 200 mg/kg + ATG + TBI (1) | Positive $CD34^+$ selection | None | 4/4 | 3–9 months in 4/4 Further remission maintained at 9 and 20 months in 2/4 | Burt *et al* (1999) |
| Hobart | 3 | Cy + G-CSF (2), BM (1) | Cy 200 mg/kg | None | None | 4/4 | <1 month in 4/4 | Lowenthal & Graham (2000) |
| GETH | 3 | – | – | None | None | 2/3 | – | Espigado *et al* (2003) |
| Nebraska | 2 | Cy + G-CSF | Cy 200 mg/kg + ATG | None | – | 2/2 | 6 months in 2/2 | Pavletic *et al* (2001) |
| Brussels | 1 | Cy 1·5 g/$m^2$ + Etop 300 mg/$m^2$ + G-CSF | Bu 16 mg/kg + Cy 120 mg/kg | Positive $CD34^+$ selection, negative CD4/8 cell selection | – | 1/1 | No relapse at 10 months | Durez *et al* (1998) |
| Leeds/Leicester | 1 | Cy 2 g/$m^2$ + G-CSF | Cy 200 mg/kg | Positive $CD34^+$ selection, negative CD4/8 cell selection | None | 1/1 | 9 months | Bingham *et al* (2002) |
| Perth | 1 | Cy 4 g/$m^2$ + G-CSF | Cy 200 mg/kg | None | None | 1/1 | 9 months followed by spontaneous remission lasting 2 years | Joske *et al* (1997) |
| Seoul | 1 | Cy 4 g/$m^2$ + G-CSF | Cy 200 mg/kg + ATG | Positive $CD34^+$ selection | None | 1/1 | No relapse after 6 months | Kim *et al* (2002b) |

Data from many of the patients reported in the smaller studies were included in the EBMT/ABMTR report

BM, bone marrow; PBSC, periphral blood stem cells; G-CSF, granulocyte colony-stimulating factor; Cy, cyclophosphamide; ATG, anti-thymocyte globulin; TBI, total body irradiation; ACR, American College of Rheumatology.

 

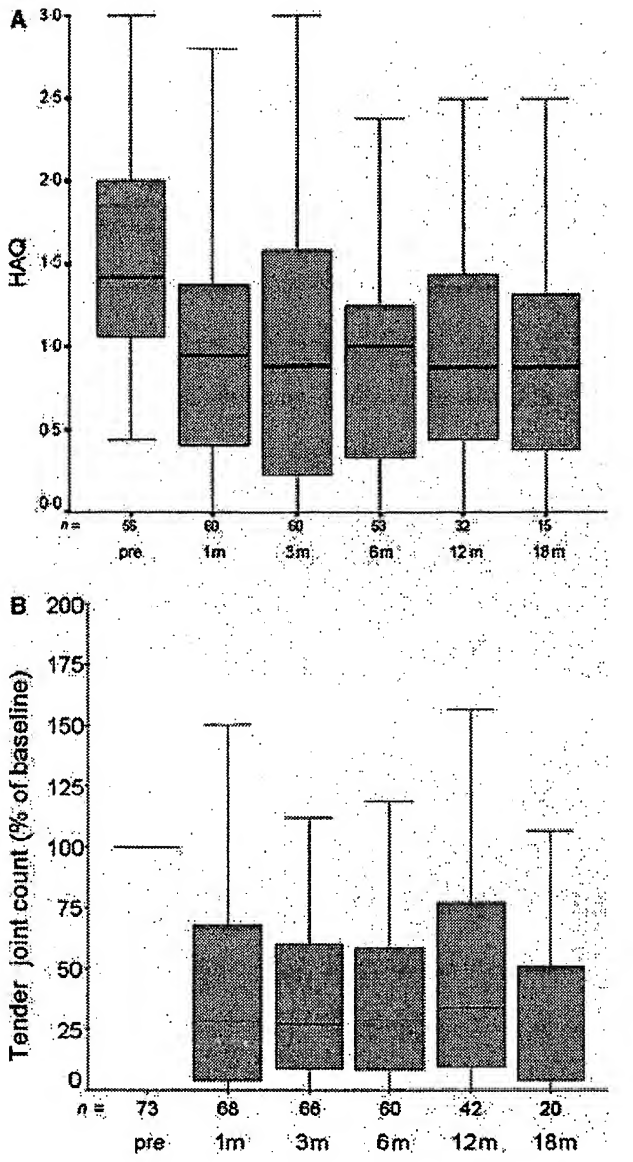


Fig 4. (A) ACR responses 6 months following autologous HSCT in RA. (B) ACR responses 12 months following autologous HSCT in RA. Both panels are from Snowden *et al* (2004b). Reproduced with permission from The Journal of Rheumatology. http://www.jrheum.com/

transplant has been recently performed in autoimmune disease with limited follow up (Burt *et al*, 2004a). A 52-year-old woman who failed a prior autograft 4 years earlier subsequently failed to respond to infliximab, methotrexate, leflunomide, prednisone, and oral and intravenous cyclophosphamide. She underwent non-myeloablative stem cell transplantation with fludarabine, cyclophosphamide and alemtuzumab followed by $CD34^+$ selected allogeneic transplant. Twelve months after the procedure she has had no infections apart from a single episode of shingles and rheumatoid symptoms have resolved. Chimaerism studies have shown increasing mixed chimaerism to 35% at 10 months.

There remains a need for autologous HSCT to be developed further. Given the responses to salvage DMARDs, the introduction of maintenance treatment early in the post-transplant course seems logical. Alternatively, there are grounds for exploring dose intensification, although this will inevitably be associated with greater toxic risks.

Post-transplant maintenance has been the focus of the EBMT/EULAR Autologous Stem Cell Transplantation International Rheumatoid Arthritis Trial (ASTIRA), in which patients are mobilized with cyclophosphamide and then randomized to receiving either maintenance treatment with methotrexate or cyclophosphamide 200 mg/kg and unmanipulated autologous stem cell rescue followed by methotrexate maintenance. In relation to dose intensification, some studies are planned by North American investigators (Snowden *et al*, 2004a).

The main issue in developing autologous HSCT for RA, especially with respect to phase III trials and attendant power considerations, is that of recruitment. Most of the early studies in autologous HSCT for RA were performed prior to the availability of biological therapies in RA. In recent years, such therapies, particularly blockers of TNF-α have become established as safe and highly effective treatments for resistant RA. There remains a significant proportion (25%) of treatment failures (Emery & Buch, 2002). The potential use of autologous HSCT is limited to these relatively rare patients, although it is arguable that such patients will also be candidates for other biological therapy.

Recruitment to clinical trials of autologous HSCT is therefore likely to be limited and this has been the case with the ASTIRA trial (J.M. van Laar, unpublished data). Although desirable, phase III studies may be ultimately impractical in terms of recruitment of sufficient numbers. It is possible that the basis for transplantation in occasional cases of resistant RA may have to depend on single arm observational data, such as the EBMT/IBMTR analysis, and improvements may have to depend on further development in phase II observational studies and analysis of larger numbers of registry data. This approach may enable the refinement of a salvage treatment for occasional patients with RA who have failed to achieve disease control with standard DMARDs and biological therapies.

### *Juvenile idiopathic arthritis*

In recent years, the treatment of children with JIA that were unresponsive to conventional anti-rheumatic drugs has been intensified considerably. Potent immunosuppressive drugs have been introduced earlier in attempts to suppress joint inflammation in those children that did not respond to non-steroidal anti-inflammatory drugs (NSAIDs). However, most paediatric rheumatologists have looked after children with JIA, particularly systemic JIA, who have not responded adequately to such treatment. Clearly the introduction of biological agents [such as anti-TNF treatment and anti-interleukin 6 (IL6) receptor treatment] for the treatment of DMARD-resistant JIA

has proven to be of great value (Lovell *et al*, 2000; Yokota *et al*, 2003). Early experience with the anti-TNF drugs also suggests that a significant proportion of children with systemic JIA are likely to remain resistant to these therapies (Quartier *et al*, 2003). Children with this refractory form of JIA not only develop severe morbidity, and significantly impaired quality of life, both from the disease, and from drug toxicities, but they have a significantly increased mortality rate (Petty, 1999; Spiegel *et al*, 2000).

The first reported use of autologous HSCT in JIA was in children with the most severe and longstanding systemic disease, with much irreversible erosive joint destruction already present (Wulffraat *et al*, 1999). Since this report more than 52 cases have been reported by nine paediatric bone marrow transplant units and registered in the EBMT database. Thirty-four of these were analysed in detail (De Kleer *et al*, 2004a). The clinical characteristics prior to ASCT in all children were a polyarticular course often complicated by erosions, osteoporosis and stunted growth. In addition, all children with systemic JIA suffered periods of spiking fever, exanthema and severe steroid-related side-effects. They were all corticosteroid-dependent and resistant to high dose parenteral methotrexate. Ten of the 34 children had failed treatment with anti-TNF therapy. BM was used as the stem cell source in 25 patients and PBSC collection using cyclophosphamide (1·5–3 g/m$^2$) and G-CSF (10 Bg/kg/d) was performed in nine patients. Most conditioning protocols were ATG and cyclophosphamide based. Nineteen patients also received ATG and low dose TBI (4 Gy, single fraction) on day −1.

The results of autologous HSCT were impressive with a prolonged drug-free follow-up of 6–60 months (Fig 5). Fifty percent of the JIA patients (17 of 31) are still in drug-free remission of the disease (with an ACR of 70) and have gained a remarkable improvement in their quality of life. One quarter of the patients (seven of 34) showed a transient or mild relapse of active arthritis, requiring treatment with corticosteroids and methotrexate. Four cases (13%) developed a persistent relapse of arthritis. Using the preliminary definition of improvement in juvenile arthritis developed by Giannini *et al* (1997), 17 of 34 patients showed a drug-free improvement of more than 50% after 4–60 months of follow-up, with a marked decrease in the scores of the Childhood Health Assessment Questionnaire, the physicians global assessment, and in swollen joint count. The measurement of limitation of movement, which largely reflects permanent joint destruction, did not, as expected, change. The erythrocyte sedimentation rate (ESR), C reactive protein and haemoglobin levels returned to near normal values with 6 weeks.

In two of these patients the ESR increased again after 3 months, with mild and transient synovitis of the hip and knee, following a varicella zoster in one and tonsillitis in the other. A relapse was noted in seven children 18 months after HSCT. Some of these relapses have been mild with oligoarthritis and sporadic fever, which could be controlled easily with a 3-month course of low dose prednisone and NSAIDs.

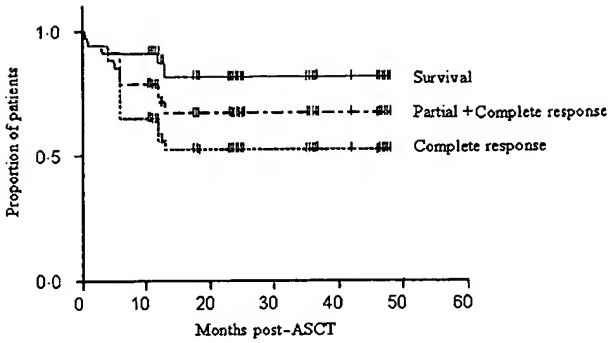


Fig 5. Responses following autologous HSCT in JIA. From De Kleer *et al* (2004a). Reproduced with permission from the BMJ Publishing Group.

Even these seven patients showed a 30% improvement in their disease. Four children were resistant to HSCT and showed a persistent recurrence of disease that was as severe as that prior to transplant.

Four patients died, with the cause of death principally the result of infection consequent on bone marrow suppression. In three of these children haemophagocytosis [also known as macrophage activation syndrome (MAS)], a well known complication of systemic JIA, reflecting marked macrophage activation because of loss of T-cell control, and perhaps an underlying abnormality of macrophage function, was also present (Wulffraat *et al*, 2003). This complication was preceded by infections including EBV reactivation and disseminated toxoplasmosis. Careful monitoring for early signs of MAS (fever, hepatomegaly, cytopenias, clotting disorders, high ferritin values), early treatment with steroids and ciclosporin, and exclusion of patients with spiking fever just before the transplant have now been added to current protocols to avoid MAS.

### *Systemic lupus erythematosis*

Systemic lupus erythematosis is a multi-system, inflammatory disorder that may include nephritis, serositis, pneumonitis, cerebritis, vasculitis, anti-phospholipid antibody syndrome with venous and vascular thrombi, arthalgias, myalgias, cutaneous symptoms. Mortality from SLE has markedly decreased because of more aggressive anti-inflammatory and immunosuppressive treatments. With the introduction of high-dose corticosteroids, addition of cyclophosphamide (intravenous pulse cyclophosphamide of 500–1000 mg/m$^2$ per month) and azathioprine, the 5-year survival in severe lupus nephritis has improved to 80%. Since 1994 more than 96 adults and two children affected with SLE were treated with HSCT, as reported to the EBMT and ABMTR (Table VIII). In adults inclusion criteria have included cyclophosphamide-resistant class III or IV glomerulonephritis (using dosages of 500–1000 mg/m$^2$/monthly i.v.), uncontrollable vasculitis (lungs, brain, heart) or transfusion-dependent cytopenias.


 

Table VIII. Phase I/II studies evaluating autologous HSCT for systemic lupus erythematosis.

| Institution | No. of patients | Mobilization regimen | Conditioning regimen | Graft manipulation | Unexpected toxicity | Response | Time to relapse | Reference |
|---|---|---|---|---|---|---|---|---|
| EULAR/EBMT | 53 | Cy + G-CSF (93%) | Various | Positive $CD34^+$ selection (42%) | 12% TRM | 33/50 by 6 months | 10/31 relapsed after 3–40 months | Jayne *et al* (2004), Tyndall *et al* (2001) |
| Chicago | 17 | Cy 2 g/m$^2$ + G-CSF (93%) | Cy 200 mg/kg + ATG + MP | Positive $CD34^+$ selection | 2/15 required ventilation for volume overload 4/15 required dialysis for fluid retention | 15/15 | 2/15 relapsed 13/15 maintained remission at median of 36 months | Traynor *et al* (2000, 2002), Burt *et al* (1997), Traynor & Burt (1999) |
| Novosibirsk | 6 | BM (4), Cy + G-CSF PBSC (2) | Various | None | 3/6 deaths due to TRM | 3/3 | None at 6–42 months | Lisukov *et al* (2004) |
| Berlin | 3 | Cy 2 g/m$^2$ + G-CSF | Cy 200 mg/kg + ATG | Positive CD34 + selection | None | 3/3 | None at 10–19 months | Rosen *et al* (2000) |
| Utrecht | 2 | BM | Cy 200 mg/kg + ATG + TBI | Negative CD2/3 selection (1) Positive $CD34^+$ selection (1) | None | 2/2 | None at 9–12 months | Wulffraat *et al* (2001) |
| Genova | 1 | BM | Thiotepa 100 mg/kg + Cy 100 mg/kg | T-cell depletion | None | 1/1 | None | Marmont *et al* (1997) |
| Vienna | 1 | Cy 2 g/m$^2$ + G-CSF | Cy 200 mg/kg + ATG | Positive $CD34^+$ selection | None | 1/1 | None at 21 months | Brunner *et al* (2002) |
| Göteborg | 1 | Cy 4 g/m$^2$ + G-CSF | Cy 2470 mg/m$^2$ + TBI | Positive $CD34^+$ selection, negative CD4/8 + 19 selection | None | 1/1 | None at 15 months | Trysberg *et al* (2000) |
| Palermo | 1 | – | Cy + ATG + Pred | – | None | 1/1 | None at 8 months Persistent positive ANA + DAT | Musso *et al* (1998) |
| Paris | 1 | Cy 4·5 g/m$^2$ + G-CSF | BEAM | Positive $CD34^+$ selection | None | 1/1 | None at 1 year ANA + anti-SSA antibodies reappeared at 9 months | Fouillard *et al* (1999) |
| Texas | 1 | Cy 2 g/m$^2$ + G-CSF | Cy 200 mg/kg + ATG + MP | Positive $CD34^+$ selection | Secondary graft failure + died day +62 from disseminated fungal infection | – | – | Shaughnessy *et al* (2001) |

Data from many of the patients reported in the smaller studies were included in the EULAR/EBMT report (Jayne *et al*, 2004).
BM, bone marrow; PBSC, periphral blood stem cells; G-CSF, granulocyte colony-stimulating factor; Cy, cyclophosphamide; ATG, anti-thymocyte globulin; TBI, total body irradiation; MP, methyl prednisolone; TRM, treatment-related mortality; BEAM: BCNU (carmustine), etoposide, cytarabine, melphalan; Pred, prednisolone; ANA, anti-nulcear antibodies, DAT, direct antiglobulin test.

After a series of case reports published since 1997, both the EBMT and ABMTR have now published retrospective surveys on the long-term outcome of SLE after HSCT (Table VIII).

A recent survey (Jayne *et al*, 2004) reported the EBMT experience with 53 SLE patients treated by ASCT in 23 centres. Patients were mostly female (83%), and had a mean of 6 years disease duration. Ethnic origin was not reported. They had a median of seven ACR diagnostic criteria for SLE present just prior to transplant, including lupus nephritis in 65%. Cyclophosphamide (50 mg/kg/d i.v. for 4 d) and ATG (2·5–5 mg/kg/d for 4 d) were used most often as the conditioning regimen. In a minority of cases lymphoid irradiation or methylprednisolone pulses were also used. Follow-up after HSCT was 26 months.

Remission of disease activity [SLE Disease Activity Index (SLEDAI) <3] was seen in 33/50 evaluable patients by 6 months, of which 10/31 subsequently relapsed after 6 months (3–40). Renal and central nervous system lupus activity also improved with a concomitant decrease in anti-dsDNA titres and increase in complement. Relapse was associated with pre-ASCT negative anti-double stranded DNA antibody ($P = 0{\cdot}015$) and normal complement levels ($P = 0{\cdot}034$). Seventy percent of patients had an immunosuppressive or biological agent re-introduced after HSCT but was a predetermined component of the transplant strategy in 40%. Impressively, it was possible to fully withdraw prednisolone therapy in 36% of patients. There were 12 deaths after 1·5 (0–48) months, of which seven (12%; 95% confidence interval, 3–21) were related to the procedure.

Jayne *et al* (2004) concluded that TRM and disease relapse appeared high. Long-term remission was associated with higher serological activity and may be improved by the use of long-term immunosuppressive therapy post-HSCT. In the EBMT database, which presently contains 420 patients, the patients with SLE have the highest TRM.

These results appear to be inconsistent with data published by Traynor *et al* (2002) reporting a single centre experience of 15 patients with SLE. Patient sex, age, disease history and previous treatment appeared comparable with those in the EBMT survey. Here, only two of 15 patients had recurrence of lupus nephritis. In a total of 34 SLE patients transplanted by the Chicago group, there was no reported mortality (Burt *et al*, 2003c). They did report five deaths after mobilization or just before HSCT (Traynor *et al*, 2002). It may be that patient selection can account for this difference.

Using the same dose of cyclophosphamide (200 mg/kg), Petri *et al* (2003) reported good results without add back of stem cells in 15 patients with SLE. Indeed, it is by no means proven that re-infusion is critical to the immune reconstitution process, or that it merely shortens the aplasia.

### *Crohn's disease*

The aetiology and pathogenesis of Crohn's disease are incompletely understood, although the common denominator is a loss of tolerance of the immune system at the gastrointestinal level, with an overactive T-helper 1 immune response. In addition to environmental agents, including intestinal flora, genetic factors are also important and mutations of the NOD2-gene influencing immune reactions against bacterial antigens are also implicated (Podolsky, 2002; Shanahan, 2002).

The activity of Crohn's disease can be controlled in many patients by the use of immunosuppressive drugs and judicious surgical intervention. Recent years have also seen the introduction of biological disease modifiers, including infliximab (Sandborn & Hanauer, 2002). Overall, life expectancy in Crohn's disease is shortened and this is attributable to the higher risks for a subset of patients in whom the disease pursues an aggressive course. These patients require relatively intense immunosuppression, frequent surgery and may run the risks of treatment hazards, including osteoporosis and consequences such as short bowel syndrome

The Chicago group have reported 10 patients (Burt *et al*, 2003d,e) with severe refractory Crohn's disease, defined as a Crohn's disease activity index greater than 250 despite treatment with TNF inhibitor, azathioprine, metronidazole and corticosteroids. The mobilization regimen consisted of cyclophosphamide (2 g/m$^2$) and G-CSF. The transplant conditioning regimen consisted of cyclophosphamide 200 mg/kg and ATG followed by CD34$^+$ selected stem cells. With 2–30 months of follow-up, no unexpected serious infective toxicity was reported. All patients achieved clinical remissions with disappearance of diarrhoea and abdominal pain and activity was reduced in all patients despite the discontinuation of immunosuppressive medications. Quality of life scores improved. Despite clinical and drug-free remissions for over 2 years in some patients, there is endoscopic and histological evidence of persistent subclinical disease (Burt *et al*, 2003d,e.)

The Freiburg group have treated one case of resistant Crohn's disease with cyclophosphamide 200 mg/kg and CD34$^+$ selected autologous stem cell transplantation (Kreisel *et al*, 2003). The patient entered remission for 9 months after mobilization with cyclophosphamide 4 g/m$^2$. After relapse, autologous transplant was performed with only routine toxicity and resulted in complete clinical, endoscopic and histological remission off immunosuppressive treatment. The remission continued for 9 months without any immunosuppressive treatment, at which point endoscopy showed early aphthous lesions and low-grade histological features without symptoms. The patient was commenced on prophylactic prednisone and thioguanine and has remained in complete remission for a further year (Kreisel *et al*, 2003; Potthoff *et al*, 2004). Preliminary data on a further patient with severe, resistant Crohn's disease in Sheffield, has confirmed a good response with acceptable toxicity at 6 months after commencing treatment (J.A. Snowden, unpublished data).

In summary, early results are encouraging in Crohn's disease. Despite the condition predominantly affecting the bowel and potential risks from suppurative manifestations, it seems possible to administer intensive immunosuppression


 

without the development of major infective problems. Clearly, the number of cases reported remains relatively limited and, as with other diseases, composite analysis of greater numbers of cases would be useful, perhaps using the registries. Ultimately, this approach would benefit from randomized controlled trial data, although recruitment of sufficient patients to such trials would be a necessary prerequisite.

### *Immune cytopenias*

Early experience of autologous transplantation in immune cytopenias was generally disappointing. In Cardiff, remissions were achieved in two patients for over 1 year, but relapse occurred soon after (Lim *et al*, 1997). Two other patients failed to show any lasting response (Skoda *et al*, 1997; Marmont *et al*, 1998).

However, in a further series, 14 patients with ITP, including five with Evans' syndrome, who had failed prior therapies including corticosteroids, splenectomy, intravenous immunoglobulin and immunomodulatory drugs were treated with cyclophosphamide 200 mg/kg and $CD34^+$ selected autologous rescue (Huhn *et al*, 2003). After 9–42 months maximum follow-up, there were no early transplant-related deaths. Six of 14 patients obtained durable complete responses of thrombocytopenia for 9–42 months, defined as platelet counts $>100 \times 10^9$/L without other therapy. Two of five patients with Evans' syndrome also achieved remissions of haemolysis (Huhn *et al*, 2003). Interestingly, post-transplant relapse appeared to be more sensitive to previously ineffective immunomodulation.

In the largest study, recently reported from the EBMT (Passweg *et al*, 2004), 27 patients with autoimmune haemolytic anaemia (five patients), Evans' syndrome (two patients), immune thrombocytopenia (12 patients), pure red cell aplasia (four patients), pure white cell aplasia (one patient) and thrombotic thrombocytopenic purpura (three patients) received an autologous HSCT following a variety of (mainly) cyclophosphamide-based conditioning regimens. Of 26 evaluable patients followed up for a median of 43 months, nine achieved a continuous partial or complete remission and six obtained a transient response. However, three died of treatment-related causes, one died of disease progression and seven did not respond.

More encouraging results have been observed with allogeneic transplantation, albeit with significant toxicity. In Salt Lake City, an HLA-identical sibling cord blood transplant resulted in normalization of platelet counts and loss of transfusion requirement in a 5-year-old child with refractory Evans' syndrome. Sadly, the patient died of hepatitis at 9 months post-transplant (Raetz *et al*, 1997). In a 28-year-old man with refractory Evans' syndrome, HLA-identical sibling transplant resulted in a remission for over 30 months (Oyama *et al*, 2001). In the EBMT series (Passweg *et al*, 2004), nine patients with autoimmune haemolytic anaemia (two patients), Evan's syndrome (five patients), pure red cell aplasia (one patient) and pure white cell aplasia (one patient) received an allogeneic HSCT. Of seven evaluable patients, five were maintained in a continuous remission at a median follow-up of 41 months, one patient had a transient response but subsequently died of progressive disease and one died of treatment-related complications.

The use of incremental doses of donor lymphocyte infusions to restore remission of Evans' syndrome following relapse after allogeneic transplantation has also been described (Marmont *et al*, 2003).

### *Other inflammatory conditions*

Other autoimmune and inflammatory diseases treated with HSCT have included Wegener's granulomatosis and other vasculitides, polymyositis (Baron *et al*, 2000; Bingham *et al*, 2001a), mixed connective tissue disease (Myllykangas-Luosujarvi *et al*, 2000), polyarteritis nodosa (Wedderburn *et al*, 2001), polychondritis (Rosen *et al*, 2000), Behcet's syndrome (Hensel *et al*, 2001; Rossi *et al*, 2004), pemphigus vulgaris (Hayag *et al*, 2000)(Table IV). At present such cases are anecdotal and broad generalizations regarding efficacy cannot be made.

## Mechanisms of response of autoimmune disease following HSCT

There has been much speculation regarding the nature of mechanisms of response of autologous and allogeneic transplantation in autoimmune disease. It has been a longstanding hope that the study of changes in peripheral blood in association with remission and relapse might provide insights into the pathophysiology of autoimmune diseases. Perhaps, unfortunately, terms such as 'resetting the immunostat', 'rebooting the immune system', 'turning back the immunological clock' and 'debulking of inflammatory load' have become accepted, and, despite opportunities and the availability of a highly developed array of techniques for studying immune reconstitution post-transplant (Peggs & Mackinnon, 2004), much work remains to be carried out before these terms have a robust scientific definition. The first effect is likely to result from the eradication of autoreactive T cells and memory cells because of the direct lymphoablative effects of drugs used in the conditioning regimen. Later, there may be an effect of altered immune reconstitution (see below).

The first stage of any transplant procedure involves a conditioning or preparative regimen. Clearly, any intensified dose of cytotoxic chemotherapy or radiotherapy, coupled with cell-specific therapeutic antibodies, will result in an instantaneous reduction in inflammatory tissue by cell death. The process is non-specific and recruitment of inflammatory cells will be restricted at least for 2–3 months until their ablated bone marrow source reconstitutes with cells derived from the graft. An example would be rheumatoid synovium, a vascular proliferating tissue, where temporary clinical improvement

was correlated with reduction of macroscopic and microscopic synovitis with arthroscopic biopsy (Bingham *et al*, 2002). There may be a dose response aspect, as low level inflammation persisted during the period of clinical quiescence.

Engraftment is followed by a period of immune reconstitution, which differs markedly between autologous and allogeneic HSCT. There will be differences relating to source of stem cells (bone marrow, peripheral blood or cord blood), graft manipulation or other T-cell depletion methods, and the type of conditioning regimen used. Thymic function is also important, with activity dependent on age and potentially on the type of autoimmune disease. The use of post-transplant therapies, either as GVHD prophylaxis or maintenance/salvage treatments is also likely to influence immune reconstitution (van Laar, 2000).

For autologous HSCT, immune reconstitution studies in autoimmune diseases confirm similar patterns of immune recovery to that seen in malignancy. These include significant reductions in numbers and function (up to 12 months) of CD4 cells and relatively rapid regeneration of CD8 and natural killer cells (Rosen *et al*, 2000; De Kleer *et al*, 2004a).

Most CD4 cells are $CD45RO^+$ cells expanded from the peripheral pool, which may include a proportion of memory cells. There is also slow but gradual thymic reconstitution of CD4 CD45RA naïve cells and T-cell receptor rearrangement excision circles, but despite this, initial hopes that autologous HSCT would result in a re-educated immune system, with thymic or extrathymic deletion of autoreactive T-cell clones, perhaps similar to tolerization of an allogeneic graft, have not been realized in practice. The network of T-regulatory cells (Tregs) provides the mechanisms that modulate or down-regulate immunological responses. The most important of these Tregs are identified by expression of CD25 and transcription factor FoxP3 (Shevach, 2000; Fontenot *et al*, 2003). Studies on $CD4^+$ $CD25^+$ Tregs in human disease are still limited. In JIA, studies are underway to correlate the number of $CD4^+$ $CD25^+$ Tregs with disease course (De Kleer *et al*, 2004b).

Interestingly, there are many examples of occurrence of autoimmune diseases with onset after autologous transplant, such as ITP, autoimmune thyroiditis and psoriasis. It is presently unknown how errors in immunoreconstitution attribute to this.

Recovery of specific cell subsets at the time of relapse or flare has been observed with the hope of identifying specific pathogenetic cells. To date there has been little consistency between studies, and, active tissue inflammation does not necessarily correlate with peripheral blood counts (Bingham *et al*, 2000; Moore *et al*, 2002). The failure to destroy potentially autoreactive memory cells might explain the inability to eradicate the autoimmune diathesis, at least with the low intensity regimens, and it is possible that improved responses with higher doses are attributable to more complete eradication of the peripheral pool of lymphocytes. Interestingly, T-cell numbers and repertoire returned to normal in a long-term remitter who received high-intensity myeloablative treatment and highly selected autologous graft. In RA, B-cell recovery was associated with post-transplant relapse, similar to observations with alemtuzumab. Post-transplant salvage treatment with rituximab was effective in abrogating disease flare in these patients (Snowden *et al*, 2004a).

Allogeneic transplantation is not only associated with the replacement of a lympho-haematopoietic system from an unaffected donor, but also profound and protracted immunosuppression which lasts decades. Evidence for a 'GVA effect' with allogeneic SCT has been supported by animal studies as discussed earlier and by clinical data. Recently, the use of incremental doses of donor lymphocyte infusions to restore remission of Evans' syndrome provides proof of principle (Marmont *et al*, 2003).

## Conclusions and future directions

Over the last decade, the use of HSCT in severe autoimmune diseases has moved from the laboratory to the clinic. Significant responses have been seen in many treatment-resistant inflammatory disorders. Relapse seems to be common following autologous HSCT, but early observations suggest restored sensitivity to previously ineffective therapy. There is an analogy here with myeloma and other low-grade lymphoproliferative disorders, which may be controlled, but rarely cured, with autologous HSCT, and then 'controlled' with maintenance therapy. More durable responses have been observed following allogeneic transplantation, which offers the additional potential advantage of eradication of residual recipient autoreactive cells by a GVA reaction.

Most patients have been selected for HSCT because they were considered refractory to all available conventional therapy and as such, HSCT can only be presently considered as salvage therapy, when all standard treatments have failed. Despite this, the first decade of clinical data has identified specific diseases where encouraging responses have been achieved in patients for whom supportive and palliative measures would have been the only therapeutic option. Although somewhat 'against the grain' of current trends of targeted therapy in many autoimmune diseases, the strength of HSCT may be its ability to (i) dose intensify conventional immunosuppressive treatment (e.g. cyclophosphamide); (ii) impact on a broad spectrum of targets in an aberrant immune network to restore stability; (iii) introduce the concept of cure of otherwise incurable disease by allogeneic transplantation.

In addition to efficacy, a key issue related to any form of HSCT is the toxicity and potential mortality. TRM and morbidity on the scale considered as routine in HSCT is rarely seen in other specialities managing autoimmune and inflammatory diseases, and, perhaps justifiably, the role of HSCT in autoimmune diseases has been an area of scrutiny, debate and controversy in rheumatological, neurological and gastroenterological circles for the last decade. This is not to say that such diseases are without risks of mortality and morbidity, but often

it is spread over many years, with patients ultimately accepting and adapting to increasing disability. If promising long-term results are achieved by HSCT, then substantial short-term risks need to be traded against the longer term risks of uncontrolled disease. Attempts have been made to quantify this using Markov Quality of Life Years analysis (Verburg *et al*, 2002), and this approach would be of paramount importance in diseases for which HSCT is a treatment option.

Reducing the TRM is essential to improving overall outcome. In the early years of these transplants, the TRM was unacceptably high in some diseases, possibly because patient selection was limited to end-stage patients with severely impaired vital organ function. Later results, as shown in the EBMT registry, seem to indicate a learning curve, which is probably the result of better patient selection. Modification of supportive care and conditioning protocols, including reduced intensity conditioning, should be explored. The best results of HSCT may be obtained when the procedure is performed early in the course of the disease. However, at the present time, not all autoimmune diseases have sufficiently robust indicators of poor outcome to identify specific patients in whom the risks of transplantation are justifiable.

Preliminary data on allogeneic HSCT (Raetz *et al*, 1997; McColl *et al*, 1999; Oyama *et al*, 2001; Burt *et al*, 2004a; Passweg *et al*, 2004; Snowden *et al*, 2004a) appears encouraging with respect to the potential for cure, something that has not been observed with any other therapy to date and has confirmed the earlier impression of superiority over autologous HSCT from the animal experiments and anecdotal case reports discussed above. However, for diseases for which the principle aim of treatment is to reduce disability, the substantial morbidity and mortality associated with allogeneic HSCT must be cautiously weighed against the potential for cure. The advent of reduced intensity transplantation has been associated with reduced short-term risks, and may yet offer a 'safer' transplant procedure in autoimmune diseases. The availability of an HLA-matched sibling is limited and the role of alternative donor stem cells, such as unrelated donors and cord blood, remains uncertain. The risks will be substantially higher than with autologous HSCT, and it remains to be seen whether this can be justified by the quality and durability of long-term responses.

Other broad developments in HSCT cellular therapy may benefit autoimmune diseases. Allogeneic mesenchymal stem cells may have a potent immune suppressive effect, as shown in a case of severe acute GVHD (Le Blanc *et al*, 2004). Concomitant use of mesenchymal stem cells could also abrogate the duration of neutropenia, and permit tissue repair as well as promoting the establishment of a new immune system. Improved understanding of stem cell plasticity might lead to the use of bone marrow-derived or embryonic stem cells in the repair and regeneration of the target tissues of the autoimmune and inflammatory diathesis, such as neural tissue in MS and bone and cartilage in RA (Burt *et al*, 2002). Moreover, transplantation of embryonic stem cells might generate tolerant immune systems across HLA barriers (Burt *et al*, 2004b).

It will be crucial for the future development of HSCT for autoimmune diseases to be designed and investigated in experienced centres with dual expertise in both transplantation and autoimmune disease. New strategies will require international collaboration to facilitate timely comparison with the current best standard of care in the context of well-designed randomized clinical trials.

In conclusion, through this cross-fertilization of haematology with many other specialties, especially rheumatology and neurology, HSCT may provide increasing therapeutic opportunities in otherwise refractory autoimmune diseases. Moreover, the ablation and re-building of immune systems, facilitated by the application of sophisticated techniques for tracking post-transplant immune reconstitution, may provide important insights into the aetiology and pathogenesis of autoimmune diseases.

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# Autoimmune Diabetes Is Suppressed by Transfer of Proinsulin-Encoding Gr-1$^+$ Myeloid Progenitor Cells That Differentiate In Vivo Into Resting Dendritic Cells

**Raymond J. Steptoe, Janine M. Ritchie, Lynelle K. Jones, and Leonard C. Harrison**

**The nature of the T-cell response to antigen is governed by the activation state of the antigen-presenting dendritic cell (DC). Immature or resting DCs have been shown to induce T-cell responses that may protect against the development of autoimmune disease. Effectively harnessing this "tolerogenic" effect of resting DCs requires that it be disease-specific and that activation of DCs by manipulation ex vivo is avoided. We reasoned that this could be achieved by transferring in vivo partially differentiated myeloid progenitor cells encoding a disease-specific autoantigen. With the aim of preventing autoimmune diabetes, we transferred myeloid progenitor cells encoding proinsulin into NOD mice. Bone marrow (BM) was cultured in granulocyte macrophage colony–stimulating factor (GM-CSF) and transforming growth factor-β1, a cytokine combination that expands myeloid cells but inhibits terminal DC differentiation, to yield Gr-1$^+$/CD11b$^+$/CD11c$^-$ myeloid progenitor cells and a minor population of CD11c$^+$/CD11b$^+$/CD86$^{lo}$ immature DCs. After transfer, Gr-1$^+$ myeloid cells acquired the characteristics of resting DCs (CD11c$^+$/MHC classII$^{int}$/CD86$^{lo}$/CD40$^{lo}$). Gr-1$^+$ myeloid cells generated from transgenic NOD mice that expressed proinsulin controlled by a major histocompatibility complex (MHC) class II promoter, but not from wild-type NOD mice, transferred into 4-week-old female NOD mice significantly suppressed diabetes development. The transfer of DC progenitors encoding a disease-specific autoantigen is, therefore, an effective immunotherapeutic strategy that could be applied to humans. *Diabetes* 54:434–442, 2005**

Antigen-presenting dendritic cells (DCs) determine the functional properties of the T-cells with which they interact. In the absence of activating signals to DCs, such as those mediated through toll-like receptors (1) or CD40 (2), the outcome of DC/T-cell interaction may be a state of T-cell "tolerance." The stage of differentiation, type, and/or location of DCs can determine the form of tolerance that ensues. For example, DCs in lymphoid tissues exist primarily in a "resting" state (3) and present antigen in a manner that appears to result in tolerance due to deletion of antigen-specific T-cells (2,4,5) or induction of unresponsiveness (6), while DCs in mucosal sites induce cytokine skewing and regulatory T-cells (7). Although DCs are promising immunotherapeutic tools, ideally they would target disease-specific T-cells. In the case of autoimmune diseases, this specificity could be achieved by endowing DCs with the ability to express autoantigen. A potential strategy would be to generate cells ex vivo with the phenotypic and functional properties of resting lymphoid tissue DCs, load them with appropriate antigen, and transfer them to effect T-cell tolerance in vivo. This approach is qualified, however, by the plasticity of DCs and the uncertainty of whether they would localize to microanatomic sites for tolerance induction in lymphoid tissues. Activation of DCs can occur during their preparation and manipulation ex vivo (8) or after transfer (9), which may abrogate their tolerogenic potential. This could be avoided by transfering DC progenitor cells that would then differentiate into inherently tolerogenic DCs in vivo. To this end, we generated partially differentiated myeloid cells that retained DC progenitor properties in vitro and in vivo by culture of bone marrow (BM) in granulocyte macrophage colony–stimulating factor (GM-CSF) and transforming growth factor (TGF)-β1, a cytokine combination that expands myeloid cells but inhibits terminal DC differentiation. We used the NOD mouse model of spontaneous autoimmune type 1 diabetes, in which proinsulin is a key autoantigen (10,11), to test the ability of DC progenitors encoding proinsulin to suppress the development of autoimmune disease in an antigen-specific manner.

## RESEARCH DESIGN AND METHODS

Female NODLt/Jax mice were obtained from the Walter and Eliza Hall Institute central breeding facilities. Proinsulin-NOD (PI-NOD) transgenic mice expressing mouse proinsulin II under control of the I-E$_\alpha^k$ major histocompatibility complex (MHC) class II promoter, described previously (10), were used

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BM, bone marrow; CFSE, 5(6)-carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte macrophage colony–stimulating factor; IFN, interferon; IL, interleukin; M-CSF R, macrophage colony–stimulating factor receptor; MHC, major histocompatibility complex; rm, recombinant murine; SA, streptavidin; TGF, transforming growth factor; TNF, tumor necrosis factor.

after breeding to homozygosity. Animals were used in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

**Reagents, cytokines, and antibodies.** Culture medium was RPMI-1640 supplemented with 1 mmol/l sodium pyruvate, 0.1 mmol/l nonessential amino acids (both from Gibco, Rockville, MA), 50 μmol/l 2-mercaptoethanol (Sigma, St. Louis, MO), and 10% vol/vol FCS (R10) (JRH, Lenexa, KS). Recombinant murine (rm) GM-CSF, interleukin (IL)-4, and tumor necrosis factor (TNF)-α were purchased from Peprotech (Rocky Hill, NJ). Recombinant human TGF-β1, rmM-CSF, and rmG-CSF were from R&D Systems (Minneapolis, MN). Interferon (IFN)-γ was kindly provided by Genentech (South San Francisco, CA). Fluorescein isothiocyanate (FITC)-dextran was purchased from Sigma. Antibodies directed against Gr-1 (Ly-6G and RB6-8C5), F4/80 (F4/80), CD11b (M1/70), CD11c (N418), MHC class II (10.2.16 [I-$A^{k,s7,r,f,s}$]), MHC class I (M1/42), macrophage colony–stimulating factor receptor (M-CSF R) (AFS-98), CD40 (FGK-45), B220 (RA3-6B2), CD205 (NLDC-145), CD86 (GL-1), and c-kit (ACK-2) were purified from hybridoma supernatants and used as purified mAb or conjugated in house. Streptavidin (SA)-fluorochrome conjugates (SA-FITC, SA-phycoerythrin, SA-allophycocyanin, and SA–Texas red) were from Caltag (Burlingame, CA). mAb directed to CD40 (3/23), MAC-3 (M3/84), CD13 (R3-242), CD62-l (MEL-14), CD31 (MEC13.3), CD43 (S7), CD11a (2D7), and CD49d (R1-2) were purchased from PharMingen (San Diego, CA). Anti-F4/80 was from Caltag (F4/80). Anti-mouse FIRE (6F12) (12) was provided by Dr. Irene Caminschi, Walter and Eliza Hall Institute. 5(6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes (Eugene, OR).

**BM cultures and cell transfers.** Mice were euthanized by $CO_2$ asphyxiation and femurs and tibiae removed aseptically. BM was flushed with mouse tonicity PBS/2.5% FCS. Cells were collected by centrifugation and erythrocytes removed by distilled water lysis. After washing in RPMI/10% FCS, cells were plated in 6-well plates (NUNC, Roskilde, Denmark) at $2 \times 10^6$/ml in 3 ml R-10 per well. Cultures were initially supplemented with GM-CSF (1 ng/ml) and 2 days later nonadherent cells removed. Remaining adherent cells were then cultured in GM-CSF (1 ng/ml) and TGF-β1 (2 ng/ml) for a further 3 days. After a total of 5 days of culture, nonadherent cells were harvested and washed twice in R-10. To generate GM-CSF/IL-4 BMDCs, BM cultures were prepared as described but supplemented with GM-CSF and IL-4 (1 ng/ml of each) for the entire culture period (13). In some experiments, subsets of cells were depleted with either anti–Gr-1 (RB6–8C5) or biotinylated anti-CD11c (N418) and sheep anti-rat Dynabeads or CELLection biotin-binding Dynabeads (Dynal Biotech; Carlton South, Victoria, Australia), respectively, according to the manufacturer's instructions. Alternatively, $CD11c^+$ cells were depleted with CD11c-phycoerythrin and anti-phycoerythrin magnetic beads (MACS; Miltenyi Biotec, Gladbach, Germany) on a magnetic cell sorter (AutoMACS; Miltenyi Biotec). Unless stated otherwise, to prevent engraftment of hematopoietic stem or progenitor cells that may have remained in the cultures, bulk or depleted cell populations were irradiated (2,000 rads, $^{60}Co$ source), washed twice in R-10, and resuspended in PBS before injection intravenously into 4-week-old female NOD mice. Irradiation of GM-CSF/TGF-β1–generated cells before transfer did not alter their effect on diabetes development. For bulk GM-CSF/TGF-β1–cultured BM, $2 \times 10^6$ cells were transferred intravenously. When cell subsets were depleted, the number of cells transferred was equivalent to that in unseparated bulk GM-CSF/TGF-β1–cultured BM, i.e., $1.8 \times 10^6$ $Gr\text{-}1^+$ (CD11c-depleted) cells or $0.2 \times 10^6$ $CD11c^+$ (Gr-1–depleted) cells.

**FITC-dextran uptake.** Quantitation of endocytosis by FITC-dextran uptake was performed as described (13). Briefly, cells were incubated with FITC dextran (1 mg/ml) for 2 h at 37°C or 4°C. Endocytosis was stopped by washing twice with ice-cold PBS containing 2.5% FCS and 0.02% sodium azide, and samples were maintained at 4°C for immunofluorescence staining and flow cytometry.

**Flow cytometric analysis.** Immunofluorescence staining of cells was performed as described previously (13) for flow cytometry on a FACScan (Becton Dickinson, San José, CA). Viable cells were gated on the basis of propidium iodide exclusion. For routine analyses, $1\text{–}2 \times 10^4$ live-gated events were collected. For analysis of in vivo DC phenotype, $1\text{–}2 \times 10^6$ live-gated events were collected.

**In vitro differentiation assays.** BM cultured in GM-CSF/TGF-β1 was harvested and washed twice, and $Gr\text{-}1^+$ or $CD11c^+$ cells were depleted with immunomagnetic beads as described above. After washing, the remaining $CD11c^+$ or $Gr\text{-}1^+$ cells were plated in 24-well tissue culture plates (Falcon; Becton Dickinson, Franklin Lakes, NJ) at $10^6$ cells/ml in 1 ml R-10 supplemented with recombinant cytokines (G-CSF, 2 ng/ml; M-CSF, 10 ng/ml; GM-CSF, 5 ng/ml; IL-4, 5 ng/ml; and TNF-α, 10 ng/ml) as described in RESULTS. Cultures were maintained in 5% $CO_2$ for up to 7 days.

**In vivo cell tracking and differentiation.** Cells harvested from GM-CSF/TGF-β1–supplemented BM cultures were prepared as described, labeled with 5 μmol/l CFSE (14), and injected intravenously. In some experiments, mice were anesthetized with methoxyflurane. Then, the spleen was everted through a keyhole incision and, after being injected with CFSE-labeled cells ($5 \times 10^6$ in 50 μl), gently reinserted into the peritoneal cavity. The wound was closed with surgical clips. Control mice received PBS alone. Mice were killed at defined time points after cell transfer and spleens and other tissues removed. For immunohistology, tissues were embedded in Tissue-Tek OCT freezing medium (Miles, Elkhart, IN). For flow cytometric analysis of in vivo DC development, spleen cell suspensions were prepared using collagenase/EDTA as described (13).

**Adoptive cotransfer assay for regulatory T-cells.** Adoptive cotransfer assays for antidiabetic regulatory cells were performed as described (15). Spleen cells ($2 \times 10^7$) from recipients of proinsulin-NOD or wild-type NOD GM-CSF/TGF-β1–cultured BM (test cells) and spleen cells ($2 \times 10^7$) from recently diabetic female NOD mice (diabetogenic cells) were mixed and injected intravenously into NOD.scid mice. Diabetes development was monitored as described below.

**Immunohistology.** Cryostat sections (5 μm) were cut from frozen OCT-embedded tissues, air dried, and fixed with cold 100% ethanol before immunostaining or mounting. Avidin/biotin binding sites were blocked using avidin/biotin blocking reagents (Vector, Burlingame, CA), and nonspecific protein interactions were blocked with 1% BSA. Biotinylated primary antibodies were applied at predetermined optimal concentrations for 1 h at room temperature. After washing, streptavidin horseradish peroxidase (ABC-Elite; Vector) or streptavidin Texas red was applied for a further hour. Immunoperoxidase slides were washed, and staining was developed with enzyme substrate (VectorRed [Vector] or 3,3′-diaminobenzidine, σ-Fast' [Sigma]) and counterstained with hematoxylin. Immunofluorescence slides were rinsed and mounted in antifade reagent (DAKO, Carpinteria, CA).

**Cytospins.** Cytospins were prepared in a cytofuge (Shandon, Pittsburgh, PA) and stained with Diff Quik (Lab Aids, Narrabeen, NSW, Australia) or by immunohistochemistry, as described.

**Assessment of proinsulin production.** $CD11c^+$ and $Gr\text{-}1^+$ cells were purified from day 5 GM-CSF/TGF-β1 BM as described. For GM-CSF/IL-4, whole cultures were analyzed. Cells were cultured for 24 h with cytokine (IFN-γ, 1,000 units/ml, and TNF-α, 10 ng/ml) or anti-CD40 (50 μg/ml) supplementation. Supernatants were harvested and stored at −20°C until assayed. Proinsulin production was measured using an insulin enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden). Proinsulin production was calculated as nanograms of $10^6$ cells per 24 h. The detection limit was <0.1 ng · $10^6$ $cells^{-1}$ · 24 $h^{-1}$.

**Monitoring of diabetes development.** Mice were tested weekly for urine glucose (Diastix; Bayer, Pymble, NSW, Australia), and if they were glycosuric, they were then tested for blood glucose (Accu-Chek; Roche, Castle Hill, NSW, Australia). Mice were scored diabetic when two consecutive blood glucose readings were >12.0 mmol/l, and then they were killed. Diabetes incidence was plotted as Kaplan-Meier survival curves (GraphPad Prism; GraphPad Software, San Diego, CA).

**Statistical analysis.** Student's *t* test was used for comparison of means (Microsoft Excel). One-way ANOVA with Neuman-Keul's post test was used for comparisons of multiple groups (GraphPad Prism). Statistical differences in diabetes incidence were analyzed by log-rank test (GraphPad Prism).

## RESULTS

**BM cultured in GM-CSF/TGF-β1 contains predominantly partially differentiated $Gr\text{-}1^+$ myeloid cells.** Addition of TGF-β1 to GM-CSF–supplemented BM cultures allows myeloid cell expansion but inhibits terminal differentiation of DCs (9,16). Therefore, we surmised that GM-CSF/TGF-β1 could be used to generate partially differentiated myeloid progenitors that retain DC development potential. GM-CSF/TGF-β1 BM cultures contained a mixture of cell types but were dominated by small round cells with annular or segmented nuclei (Fig. 1*A*), expressing the myeloid differentiation antigen Gr-1 (Ly-6G; Fig. 1*B*). These features are characteristic of partially differentiated myeloid cells (17). Florescence-activated cell sorter analysis confirmed the preponderance of $Gr\text{-}1^+$ cells and their expression of the myeloid marker CD11b but not the DC-specific marker CD11c (Fig. 2). $Gr\text{-}1^+$ cells expressed negligible levels of MHC class I, MHC class II, F4/80, FIRE, MAC 3, CD13 M-CSF R, and c-kit (CD117) (Fig. 2). The

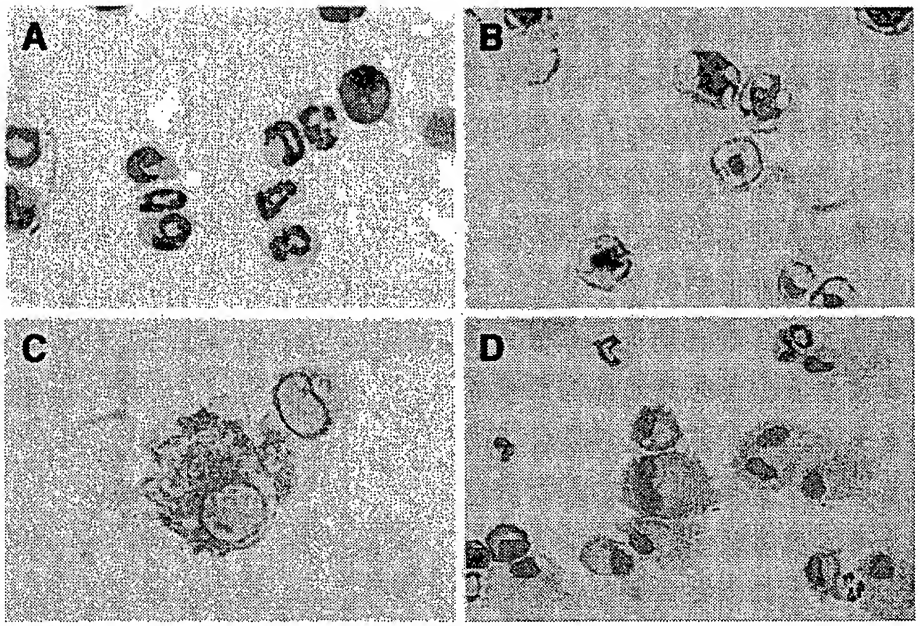


FIG. 1. BM cultured in GM-CSF/TGF-β1 containing predominantly partially differentiated Gr-1$^+$ myeloid cells. Cytospins were prepared from BM cultured in GM-CSF/TGF-β1 (*A*, *B*, and *C*) or GM-CSF/IL-4 (*D*) and stained with Diff Quik (*A* and *D*), antibodies to Gr-1 (*B*), or MHC class II (*C*).

adhesion molecules CD49d and CD11a (18,19) used by mature neutrophils to home to sites of inflammation were either not expressed or were expressed at a relatively low level, respectively, by Gr-1$^+$ cells, signifying that these cells were not mature neutrophils. Expression of the myeloid differentiation markers CD43 and CD31 (17,20,21) on Gr-1$^+$ cells was heterogenous, indicating that these cells were present in various differentiation states.

A small proportion (8.5 ± 2.1% [mean ± SD], $n$ = 20) of GM-CSF/TGF-β1 BM cells had a monocyte-like or immature DC-like appearance (Fig. 1*C*) and expressed low levels of MHC class II restricted primarily to intracellular granules (Fig. 1*C*). Florescence-activated cell sorter analysis showed that this Gr-1$^-$ fraction comprised almost exclusively cells that expressed low or intermediate levels of CD11c. In addition to MHC class I and moderate levels of MHC class II, CD11c$^+$ cells expressed F4/80, FIRE, MAC 3, CD13, and c-kit (CD117) (Fig. 2*A*), molecules expressed by immature BMDCs or DCs in vivo. CD11c$^+$ cells also expressed CD62L (L-selectin) used by DC/monocyte precursors to home from blood to lymph nodes (22). The majority of the CD11c$^+$ cells expressed M-CSF R and high levels of CD11b, as described previously for immature BMDCs from NOD mice (13).

To further define CD11c$^+$ cells from GM-CSF/TGF-β1 BM, we compared BM cultured in GM-CSF/TGF-β1 and GM-CSF/IL-4, as the latter contains a mix of phenotypically mature and immature DCs (Fig. 1*D* and online appendix available at http://diabetes.diabetesjournals.org). CD11c$^+$ cells from GM-CSF/TGF-β1 BM were phenotypically similar to the immature subset of DCs generated in GM-CSF/IL-4 (online appendix). Endocytic activity, a hallmark of functionally immature (CD11c$^+$/CD86$^{lo}$) DCs (online appendix), measured in GM-CSF/TGF-β1 BM by FITC-dextran uptake, was restricted to CD11c$^+$ cells (online appendix). In contrast to Gr-1$^+$ myeloid cells, CD11c$^+$ cells harvested from GM-CSF/TGF-β BM were more differentiated, as evidenced by their rapid development (within 2 days) into DCs in GM-CSF, IL-4, and TNF-α or into macrophages in M-CSF or GM-CSF/M-CSF. CD11c$^+$ cells rapidly died in cultures supplemented only with G-CSF (not shown). Collectively, these findings indicate that Gr-1$^+$ cells in GM-CSF/TGF-β1 BM are partially differentiated myeloid cells, whereas the minor population of CD11c$^+$ cells are phenotypically and functionally immature DCs.

**Gr-1$^+$ cells are multipotent myeloid progenitors.** To investigate their differentiation potential, Gr-1$^+$ cells isolated from GM-CSF/TGF-β1 BM were exposed to cytokines that drive different myeloid differentiation pathways. In G-CSF, Gr-1$^+$ cells retained their small rounded profile but rapidly acquired highly segmented nuclei and a higher level of Gr-1 expression characteristic of mature granulocytes (Fig. 3). Cells with DC- or macrophage-like characteristics were not detected after 2 days in G-CSF. Consistent with their short lifespan, the numbers of mature granulocytes diminished after 2 days in G-CSF (not shown). In the DC-inducing combination of GM-CSF, IL-4, and TNF-α, small numbers of DC-like cells were seen as early as 2 days, and by 7 days large numbers of cells displayed classic DC morphology (Fig. 3). Flow cytometry revealed that exposure to GM-CSF, IL-4, and TNF-α resulted in loss of Gr-1 and acquisition of CD11c expression by the majority of cells (Fig. 3). CD11c$^+$ cells were approximately equally distributed between phenotypically mature (MHC class II$^{hi}$ or CD86$^{hi}$) and immature (MHC class II$^{lo}$ or CD86$^{lo}$) DC subsets (Fig. 3). In M-CSF, Gr-1 expression was lost and adherent macrophage-like cells that retained CD11b expression and acquired low-level expression of the macrophage marker F4/80 appeared.

**Similar phenotype of GM-CSF/TGF-β1 BM cells from PI-NOD and wild-type NOD mice.** No differences were detected between cells generated from PI-NOD and NOD mice in the presence of either GM-CSF/TGF-β1 (Fig. 4) or GM-CSF/IL-4 (not shown).

**CD11c$^+$ DCs but not Gr-1$^+$ myeloid cells from PI-NOD transgenic mouse BM produce proinsulin.** CD11c$^+$ DCs generated from PI-NOD transgenic mouse BM in GM-CSF/IL-4 produced threefold more proinsulin than CD11c$^+$ DCs from GM-CSF/TGF-β1 BM (4.2 ± 2.3 vs.

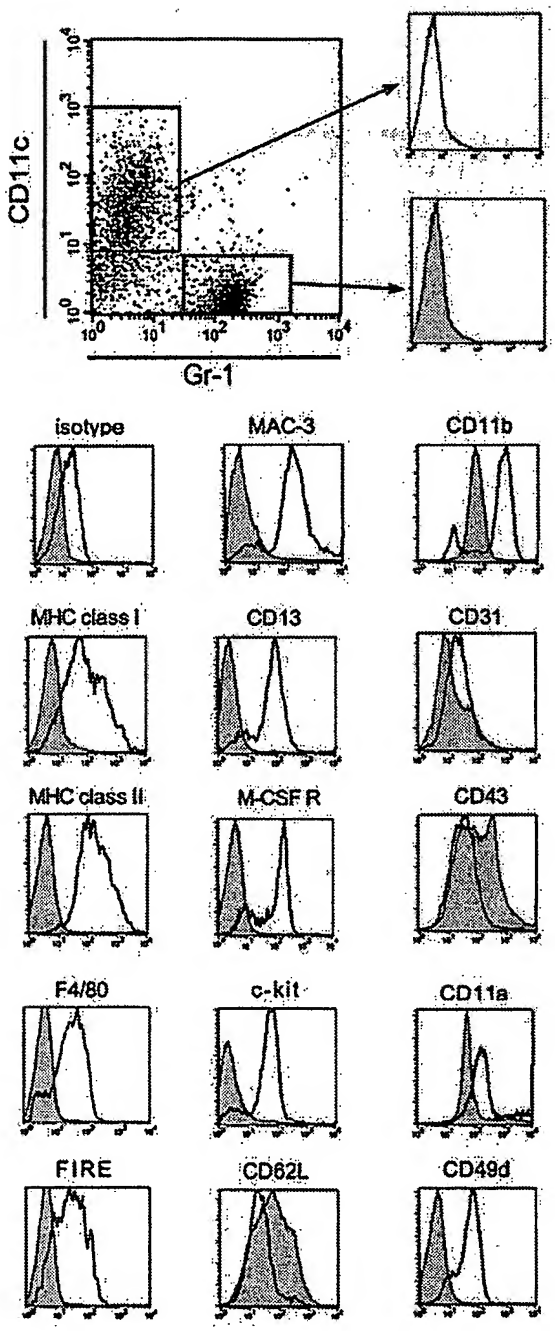


FIG. 2. Undifferentiated myeloid cells are abundant in GM-CSF/TGF-β1–cultured BM. BM was cultured in GM-CSF/TGF-β1 and cells harvested at day 5. Cell surface markers expressed on Gr-1$^+$ and CD11c$^+$ cells were analyzed using four-color flow cytometry. Upper left dot plot shows gating used for analysis of Gr-1$^+$ and CD11c$^+$ cells. Histogram overlays show Gr-1–gated (shaded) and CD11c-gated (open) cells.

1.3 ± 1.1 ng · $10^6$ cells$^{-1}$ · 24 h$^{-1}$). Cytokines that upregulate MHC class II expression (IFN-γ/TNF-α) enhanced proinsulin production by CD11c$^+$ DCs from GM-CSF/TGF-β1 cultures (to 3.4 ± 1.7 ng · $10^6$ cells$^{-1}$ · 24 h$^{-1}$, $P < 0.05$), whereas agonistic anti-CD40 mAb enhanced proinsulin production by DCs from GM-CSF/IL-4 cultures (to 8.0 ± 2.9 ng · $10^6$ cells$^{-1}$ · 24 h$^{-1}$, $P < 0.05$) but not GM-CSF/TGF-β1 cultures. This is consistent with the expression of CD40 by DCs from GM-CSF/IL-4 BM but not GM-CSF/TGF-β1 BM. Purified Gr-1$^+$ cells did not produce detectable proinsulin, even after stimulation with GM-CSF/IFN-γ/TNF-α.

**Diabetes development is suppressed by transfer of proinsulin-encoding Gr-1$^+$ undifferentiated myeloid cells.** GM-CSF/TGF-β1 BM from PI-NOD mice transferred to 4-week-old female NOD mice suppressed diabetes development (Fig. 5*A*). To identify the cell responsible for this protective effect, CD11c$^+$ or Gr-1$^+$ cells were depleted from GM-CSF/TGF-β1 BM before transfer. Depletion of Gr-1$^+$, but not CD11c$^+$, cells abolished the protective effect (Fig. 5*B*). This was then demonstrated directly and shown to be proinsulin dependent by transfer of purified Gr-1$^+$ cells from GM-CSF/TGF-β1 BM of PI-NOD mice (Fig. 5*C*). Thus, diabetes development was suppressed by transfer of Gr-1$^+$ partially differentiated myeloid cells and not by the CD11c$^+$/CD86$^{lo}$ immature DCs present in GM-CSF/TGF-β1 NOD-PI BM.

**Gr-1$^+$ myeloid cells differentiate into DCs in vivo.** Our hypothesis was that PI-encoding Gr-1$^+$ myeloid cells would protect against diabetes because they differentiated into "tolerogenic" resting DC in vivo. To examine their fate, Gr-1$^+$ myeloid cells purified from PI-NOD GM-CSF/TGF-β1 BM were labeled with CFSE to enable visualization after transfer. Two days after intravenous transfer into NOD mice, CFSE-labeled cells (~1–2 per 10× field) could be detected in cryostat sections of spleen, lung, and liver but not in peripheral blood or other tissues (thymus, pancreas, small intestine, kidney, inguinal lymph node, and pancreatic lymph node). CFSE-labeled cells in liver and lung diminished rapidly in number after 2 days but were still detected in spleen (≥1 per 10× field) at 4 and 6 days posttransfer. Because they were preferentially retained in spleen, we injected CFSE-labeled Gr-1$^+$ cells directly into spleen to examine their differentiation. One day after injection, abundant CFSE-labeled cells with a rounded profile that stained for CD11b and Gr-1, but rarely for MHC class II, were clearly visible. Three days after injection, CFSE-labeled cells were present predominantly in the T-cell areas and had developed a larger, more stellate appearance. Many continued to express CD11b but had lost Gr-1 expression, and 30–50% had acquired substantial expression of MHC class II and CD11c (Fig. 6). To determine the phenotype of the CD11c$^+$ DCs that developed in vivo, spleens were collected after 3 days for flow cytometry. CD11c$^+$ cells comprised a large proportion of the CFSE-labeled cells in the spleen (Fig. 7*A*). Three-color flow-cytometry revealed that CFSE$^+$/CD11c$^+$ DCs were almost equally distributed between the CD11c$^+$/CD8$^+$ and CD11c$^+$/CD11b$^+$ subtypes. This contrasted with the dominance of CD11c$^+$/CD11b$^+$ or CD11c$^+$/CD8$^-$ DC subtype (Fig. 7*B*) normally found in NOD mice (13). Analysis of DEC-205, normally expressed on CD8$^+$ splenic DCs, confirmed the distribution of CFSE$^+$ DCs between CD8$^+$ and CD8$^-$ subtypes (Fig. 7*B*). CFSE$^+$ DCs derived from Gr-1$^+$ progenitors exhibited a "resting" phenotype, expressing intermediate levels of MHC class II and low levels of the costimulatory/signaling molecules CD86 and CD40, identical to those of unlabeled recipient DCs (Fig. 7*C*).

**Absence of regulatory, antidiabetogenic cells after transfer of GM-CSF/TGF-β1–cultured BM.** A potential mechanism for the diabetes protective effect of Gr-1$^+$ myeloid cells is induction of regulatory, antidiabetic T-

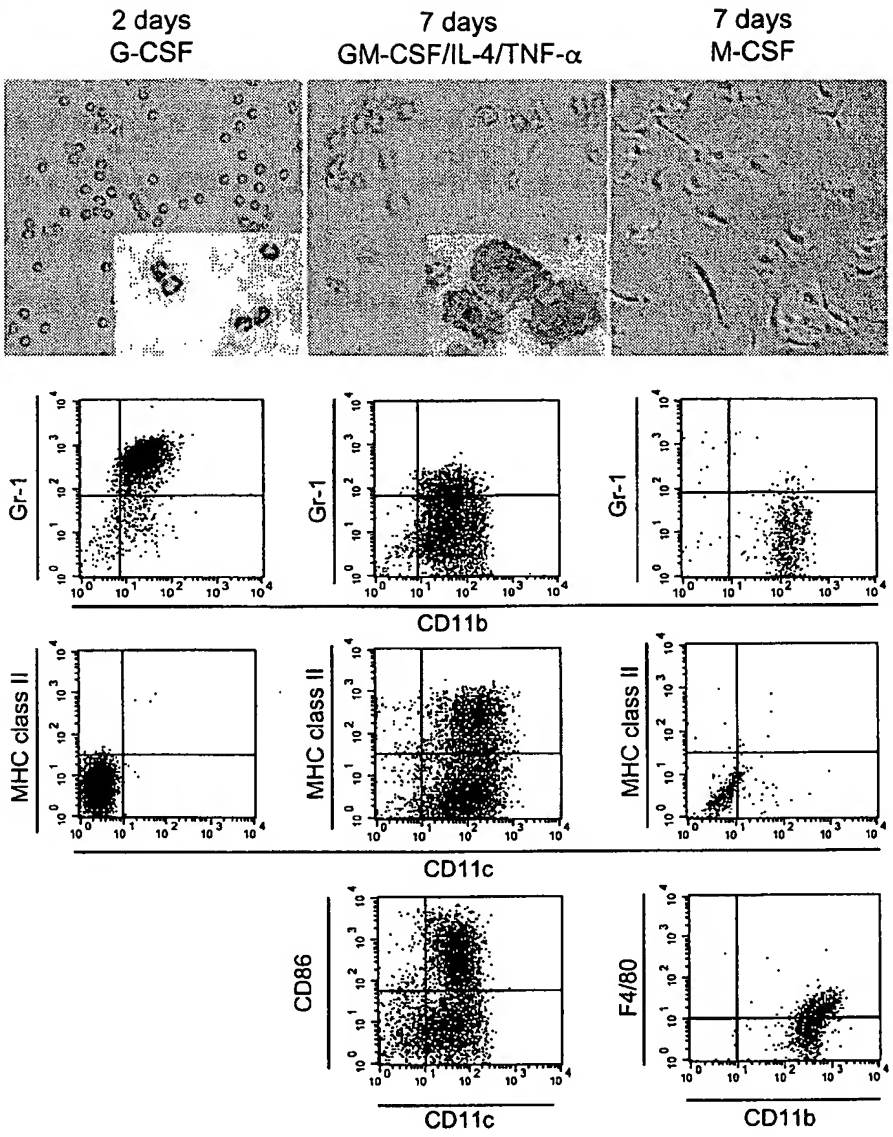


**FIG. 3. Gr-1$^+$ cells are multipotent myeloid progenitors. Gr-1$^+$ cells were purified from GM-CSF/TGF-β1–cultured BM and cultured in the presence of the cytokines shown. Photomicrographs show appearance in culture (phase contrast microscopy) or in cytospin preparations stained with Diff Quik (G-CSF) or anti-MHC class II (GM-CSF/IL-4/TNF-α). Lower panels show flow cytometric analysis of surface markers.**

cells. To test for regulatory T-cells, spleen cells from NOD mice that had received NOD or NOD-PI GM-CSF/TGF-β1 BM 4 weeks previously were cotransferred with diabetogenic spleen cells from recently diabetic female NOD mice into immunodeficient NOD.scid recipients. The proportion of mice that developed diabetes by 12 weeks after transfer was similar in recipients of spleen cells from mice that received GM-CSF/TGF-β1 BM from NOD-PI (78 ± 21%) or wild-type NOD (69 ± 32%) mice.

## DISCUSSION

Proinsulin-encoding myeloid DC progenitors generated in BM cultured in GM-CSF/TGF-β1 differentiate into resting DCs in vivo and suppress the development of autoimmune diabetes in NOD mice. Achieving expression of antigen in resting DCs by transfering antigen-encoding DC progenitors is a novel strategy for preventing autoimmune disease. Previously, the addition of TGF-β1 to GM-CSF–supplemented BM cultures was shown to inhibit the final maturation steps of DC development, leading to the generation of phenotypically and functionally immature DCs (9,16). We found that this combination of cytokines also leads to the accumulation of partially differentiated myeloid cells. While Gr-1 is routinely used as a neutrophil marker, it is expressed by a range of myeloid progenitors and, at least transiently, by monocytic cells such as those elicited to the peritoneal cavity (19), as well as by a recently described population of murine blood monocytes that exhibits DC differentiation capacity (23). Our finding that Gr-1$^+$ cells can give rise to DCs and macrophages in vitro is consistent with the observations of others (17,24). In addition, we now show that these cells also differentiate into both major subtypes (CD8$^+$ and CD8$^-$) of lymphoid tissue DCs in vivo, complementing other evidence (25,26) that myeloid-committed cells are capable of giving rise to both CD8$^+$ and CD8$^-$ DCs in vivo. Undifferentiated myeloid cells (CD11b$^+$/Gr-1$^+$/CD31$^+$) that have been termed natural suppressor cells inhibit CD8$^+$ T-cell activation by antigen-nonspecific nitric oxide–dependent mechanisms (27). A role for these myeloid suppressor cells in our experiments is excluded by their absence of MHC class II expression (27). The crucial requirement for MHC class II$^+$ progeny of Gr-1$^+$ cells for diabetes suppression was demonstrated by the lack of effect of wild-type Gr-1$^+$ cells

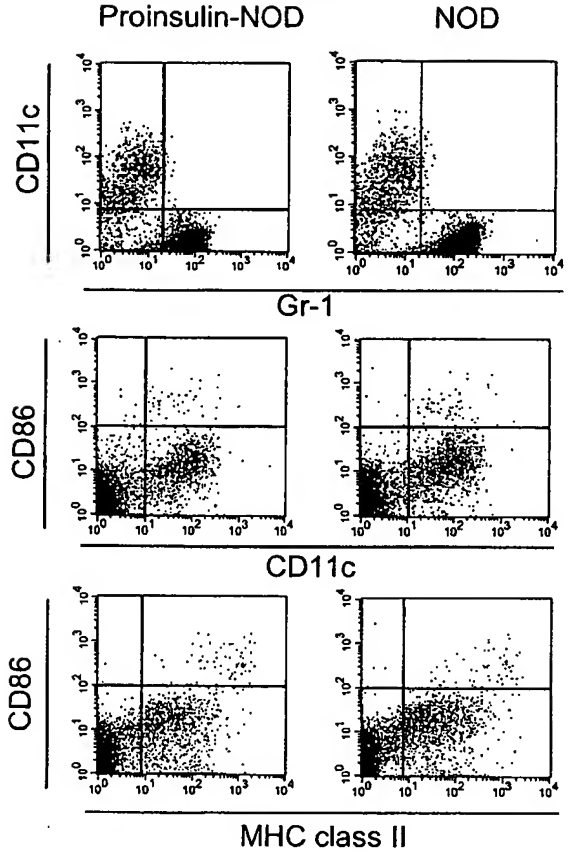


FIG. 4. Similar phenotype of GM-CSF/TGF-β1 BM cells from PI-NOD and wild-type NOD mice. BM from PI-NOD or wild-type NOD mice was cultured in GM-CSF/TGF-β1 for 5 days, cells were harvested, and surface marker expression was analyzed by flow cytometry.

without the MHC class II–driven proinsulin transgene. Additionally, the low expression of CD31 on Gr-1$^+$ cells generated in GM-CSF/TGF-β1 indicates that they are more differentiated than CD31$^+$ myeloid suppressor cells (21,28).

In contrast to proinsulin-encoding Gr-1$^+$ myeloid cells, proinsulin-encoding immature DCs were not protective. This may be because they were activated in vitro or because exposure to TGF-β1 decreases the expression of CCR7 (29), which coordinates DC migration and interaction with T-cells in secondary lymphoid tissues (30). Our findings suggest that Gr-1$^+$ myeloid progenitors may have been overlooked in previous studies examining the tolerance-inducing capacity of GM-CSF/TGF-β1–generated immature DCs (9). Because Gr-1$^+$ myeloid cells represent a large proportion of cells in BM (21), they are a potentially important source of DC progenitors that could be harnessed for tolerance induction.

The mechanism by which proinsulin-encoding myeloid progenitor cells suppress diabetes development remains unclear. Because transferred CFSE-labeled Gr-1$^+$ cells migrated primarily to spleen, liver, and lung, and not thymus, protection from diabetes is likely to be due to peripheral rather than central tolerance. While there is debate about whether specific subsets of DCs, e.g., CD8$^+$ or CD8$^-$, are "specialized" for peripheral tolerance induction, it is nevertheless clear that DCs must be in a nonactivated or resting state for tolerance to ensue

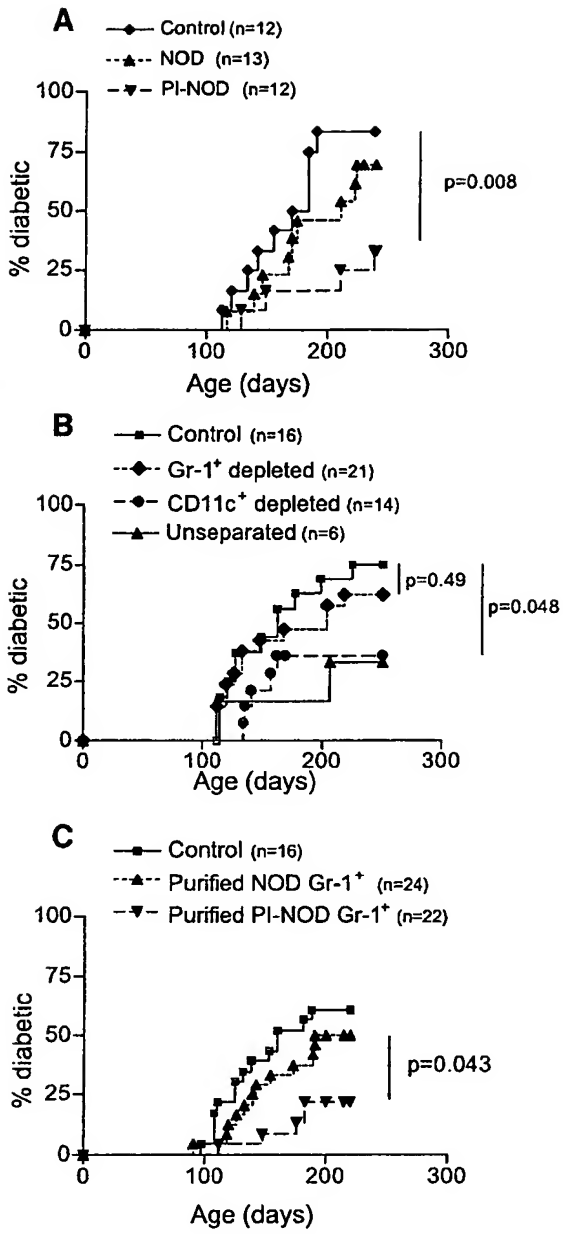


FIG. 5. Diabetes development is suppressed by transfer of proinsulin-encoding Gr-1$^+$ undifferentiated myeloid cells. *A*: GM-CSF/TGF-β1–cultured BM from proinsulin-NOD (t) or NOD (▲) mice was transferred intravenously to 4-week-old female NOD mice and diabetes development determined. Control mice received PBS. Diabetes development was significantly suppressed ($P < 0.01$) by transfer of GM-CSF/TGF-β1–cultured proinsulin-NOD but not wild-type NOD BM. Data were pooled from two separate experiments in which proinsulin-NOD and NOD cells were tested in parallel. *B*: BM from proinsulin-NOD mice was harvested from GM-CSF/TGF-β1–supplemented cultures. Gr-1$^+$ or CD11c$^+$ cells were depleted using immunomagnetic beads, the remaining cells (CD11c$^+$ or Gr-1$^+$, respectively) were transferred intravenously to 4-week-old female NOD mice, and diabetes development was compared with contemporaneous controls. Diabetes development was significantly suppressed ($P < 0.05$) by transfer of CD11c$^+$ cells depleted but not Gr-1$^+$ cell–depleted GM-CSF/TGF-β1–cultured proinsulin-NOD BM. Data were pooled from two separate experiments in which cells were tested in parallel. *C*: BM from proinsulin-NOD or NOD mice was harvested from GM-CSF/TGF-β1–supplemented cultures. CD11c$^+$ cells were depleted using immunomagnetic beads, the remaining Gr-1$^+$ cells were transferred intravenously to 4-week-old female NOD mice, and diabetes development was compared with contemporaneous controls. Diabetes development was significantly suppressed ($P < 0.05$) by transfer of Gr-1$^+$ cells from proinsulin-NOD (t) but not NOD (▲) GM-CSF/TGF-β1–cultured BM. Data were pooled from two separate experiments in which proinsulin-NOD and NOD cells were tested in parallel.

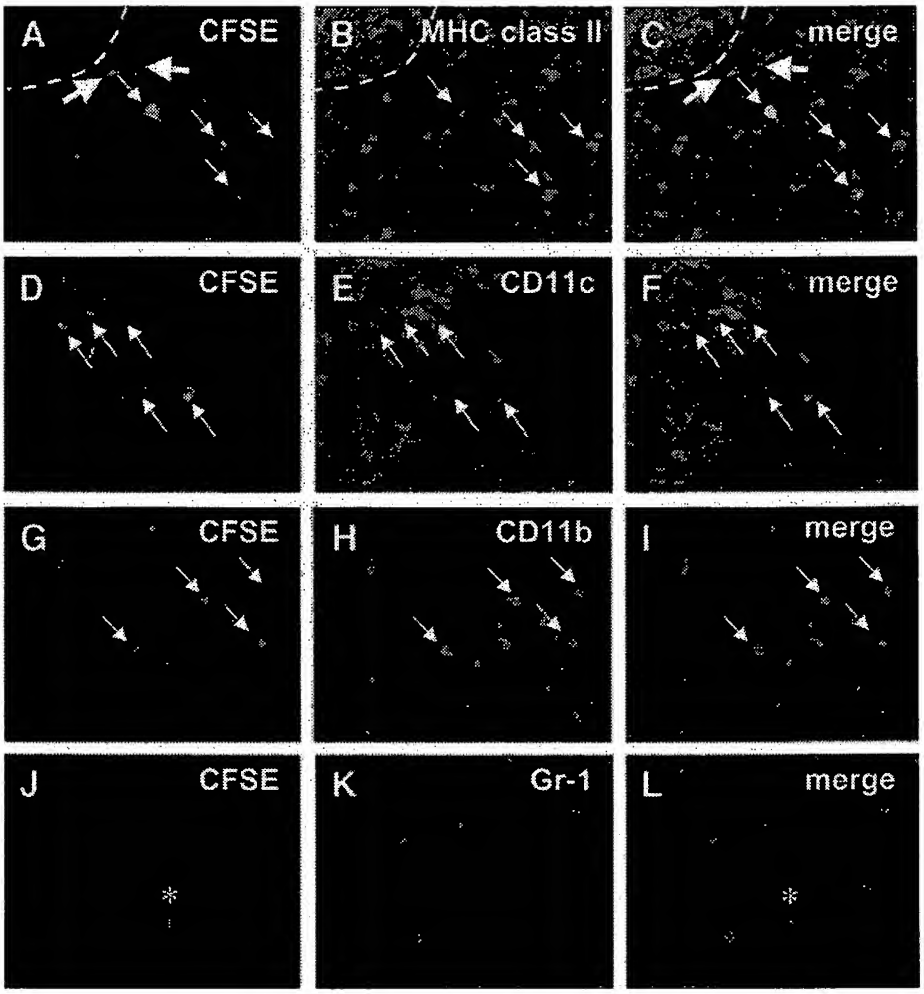


FIG. 6. Gr-1$^+$ myeloid cells differentiate to CD11c$^+$/MHC class II$^+$ DCs in vivo. Gr-1$^+$ cells were purified from GM-CSF/TGF-β1–cultured proinsulin-NOD BM, CFSE labeled, and injected directly into the spleen. After 3 days, frozen sections of spleen were stained for immunofluorescence analysis. Localization of CFSE- and antibody-labeled cells was performed by immunofluorescence microscopy. Panels show CFSE-labeled cells (*A*, *D*, *G*, and *J*) visualized with Texas red–conjugated monoclonal antibody as indicated (*B*, *E*, *H*, and *K*) and the merged images (*C*, *F*, *I*, and *L*). The dashed lines delineate the boundary of the T-cell area (below line) and B-cell area (above line). In *A*, *B*, and *C*, the thin arrows show CFSE$^+$ cells expressing MHC class II and the broad arrows show CFSE$^+$ cells not expressing MHC class II. In *D*, *E*, and *F*, arrows show CFSE$^+$ cells expressing CD11c. In *G*, *H*, and *I*, arrows show CFSE$^+$ cells expressing CD11b. In *J* and *L*, asterisks indicate CFSE$^+$ cells not expressing Gr-1. (Gr-1 cells are normally rare in the spleen.)

(2,4,5,31). Targeting antigen expression to resting as opposed to activated lymphoid tissue DCs, either by antibody-antigen conjugates or genetically (5), as achieved here for proinsulin, induces deletional tolerance and/or unresponsiveness in antigen-specific T-cells (2,4,6,31). Because both CD8$^+$ and CD8$^-$ DCs that differentiated from transferred Gr-1$^+$ myeloid progenitors exhibited a resting phenotype, we propose that diabetes protection is due to deletion or unresponsiveness of proinsulin-reactive T-cells. This is consistent with our inability to demonstrate the presence of antidiabetogenic Treg. Unfortunately, in common with others (32,33), we are unable to obtain sensitive and reproducible responses to proinsulin by NOD mouse T-cells ex vivo and therefore cannot directly test these possibilities.

Because of their costimulation dependence (34), CD4$^+$/CD25$^+$ Treg require activation by "mature" DCs to elicit regulatory function (35). Similarly, other forms of Treg in NOD mice may be most efficiently induced by mature DCs (36–38). This supports suggestions that the impaired maturation potential of NOD DCs (13,39) could lead to reduced Treg activation in vivo (40,41). In this setting, tolerance can be restored by activating DCs in vivo (42) or in an islet antigen–independent manner by adoptively transferring DCs matured ex vivo (36–38). In contrast, our findings indicate that the ability of resting DCs to induce tolerance is unaffected by the alteration of DC development in NOD mice.

The literature provides little evidence of the ability of DCs to suppress spontaneously as opposed to experimentally induced autoimmune disease. Attempts to generate tolerogenic DCs that suppress diabetes in an autoantigen-dependent manner after transfer to NOD mice have had little success. In instances where diabetes has been suppressed by DCs generated ex vivo, no requirement for presentation of β-cell antigens has been found (36–38,43–45). Our findings show that an autoantigen-dependent protective effect, an important requirement for progressing DC-based immunotherapy to the clinic, can be achieved by transferring genetically modified Gr-1$^+$ myeloid progenitors. DC progenitors encoding autoantigen driven by a differentiation stage-specific promoter have several advantages over other DC-based strategies for autoimmune disease prevention. Activation and the risk of antigen presentation that could elicit a pathogenic immune response is minimized. DC progeny express whole autoantigen protein; therefore, assumptions about epitope determinants that are necessary with peptides are avoided, as is the need for substantial quantities of pure protein autoantigen. This immunotherapeutic strategy could be applied to prevent type 1 diabetes and other autoimmune diseases in humans.

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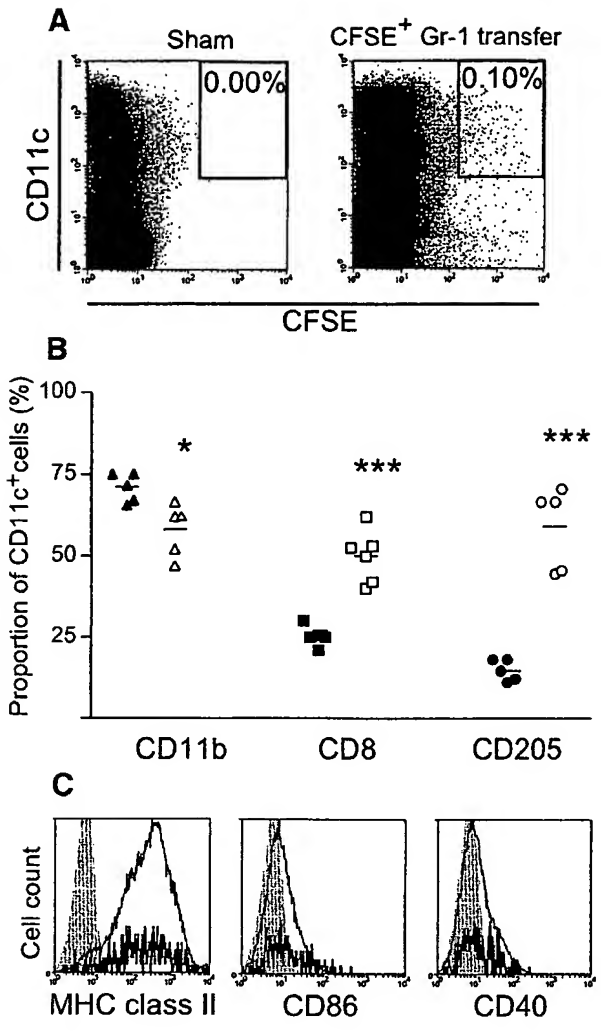


**FIG. 7. Gr-1$^+$ myeloid cell–derived DCs have a resting phenotype in vivo. *A–C*: Gr-1$^+$ cells were purified from GM-CSF/TGF-β1–cultured proinsulin-NOD BM, CFSE-labeled, and injected directly into the spleen. Three days later spleens were digested and the phenotype of CFSE-labeled Gr-1$^+$ cell-derived DCs determined by flow cytometry. *A*: Percentage of CFSE-labeled cells that express CD11c in the spleen of mice receiving no cells (left) or CFSE-labeled Gr-1$^+$ cells (right). Similar results were obtained in six mice analyzed in two separate experiments. *B*: Proportion of CFSE$^-$ (closed symbols) or CFSE$^+$ (open symbols) CD11c$^+$ DCs expressing phenotypic markers of splenic DC subtypes. Cells from individual mice were gated on CFSE$^+$ or CFSE$^-$ CD11c$^+$ cells for analysis. Data for each phenotypic marker are pooled from two separate experiments. *Significantly less than CFSE$^-$ DCs ($P < 0.05$); ***significantly greater than CFSE$^-$ DCs ($P < 0.001$). *C*: Histograms depicting expression of MHC class II, CD86, and CD40 on CFSE$^-$ (thin line) or CFSE$^+$ (bold line) DCs in recipients of Gr-1$^+$ myeloid cells. Cells from individual mice were gated on CFSE$^+$/CD11c$^+$ or CFSE$^-$/CD11c$^+$ DC for analysis. Solid gray histograms depict background fluorescence of unstained cells. Similar results were obtained in six mice in two separate experiments.**

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# Increased Generation of Dendritic Cells from Myeloid Progenitors in Autoimmune-Prone Nonobese Diabetic Mice[1]

Raymond J. Steptoe, Janine M. Ritchie, and Leonard C. Harrison[2]

**Aberrant dendritic cell (DC) development and function may contribute to autoimmune disease susceptibility. To address this hypothesis at the level of myeloid lineage-derived DC we compared the development of DC from bone marrow progenitors in vitro and DC populations in vivo in autoimmune diabetes-prone nonobese diabetic (NOD) mice, recombinant congenic nonobese diabetes-resistant (NOR) mice, and unrelated BALB/c and C57BL/6 (BL/6) mice. In GM-CSF/IL-4-supplemented bone marrow cultures, DC developed in significantly greater numbers from NOD than from NOR, BALB/c, and BL/6 mice. Likewise, DC developed in greater numbers from sorted (lineage$^-$IL-7R$\alpha^-$SCA-1$^-$c-*kit*$^+$) NOD myeloid progenitors in either GM-CSF/IL-4 or GM-CSF/stem cell factor (SCF)/TNF-$\alpha$. [$^3$H]TdR incorporation indicated that the increased generation of NOD DC was due to higher levels of myeloid progenitor proliferation. Generation of DC with the early-acting hematopoietic growth factor, flt3 ligand, revealed that while the increased DC-generative capacity of myeloid-committed progenitors was restricted to NOD cells, early lineage-uncommitted progenitors from both NOD and NOR had increased DC-generative capacity relative to BALB/c and BL/6. Consistent with these findings, NOD and NOR mice had increased numbers of DC in blood and thymus and NOD had an increased proportion of the putative myeloid DC (CD11c$^+$CD11b$^+$) subset within spleen. These findings demonstrate that diabetes-prone NOD mice exhibit a myeloid lineage-specific increase in DC generative capacity relative to diabetes-resistant recombinant congenic NOR mice. We propose that an imbalance favoring development of DC from myeloid-committed progenitors predisposes to autoimmune disease in NOD mice.** ***The Journal of Immunology*****, 2002, 168: 5032–5041.**

Insulin-dependent or type 1 diabetes (T1D)[3] results from autoimmune destruction of insulin-secreting $\beta$ cells in the pancreatic islets of Langerhans. The nature of immune dysregulation leading to $\beta$ cell destruction remains poorly understood. However, phenotypic and functional abnormalities of dendritic cells (DC) and myeloid-lineage development have previously been identified in humans at risk for or with T1D (1, 2), as well as in the most widely used animal model of T1D, the nonobese diabetic (NOD) mouse (3, 4).

While many subpopulations of DC have been described based on their anatomical location and phenotypic or functional characteristics (reviewed in Ref. 5), two broad DC subtypes have been proposed. Myeloid DC express high levels of myeloid lineage-associated markers such as CD11b, whereas the other proposed subtype expresses low levels of myeloid markers and high levels of several lymphoid-associated markers. In mice, the latter subtype is CD8$\alpha^+$DEC-205$^+$CD11b$^-$ (6) and was termed lymphoid-related DC after being shown to develop in vivo from early lymphoid precursors (7). Despite uncertainty regarding the developmental origins of these DC subtypes (8, 9), they exhibit quite distinct functional characteristics in vivo. For example, CD8$\alpha^-$ myeloid DC traffic to regional lymphoid tissues from peripheral tissue, whereas CD8$\alpha^+$ lymphoid DC appear to develop in situ in lymphoid tissues (10, 11). Administration of flt3 ligand (flt3-L) expands both DC subtypes, but GM-CSF expands only the myeloid subtype (12). While CD8$\alpha^-$ DC present soluble Ag to CD4$^+$ T cells, CD8$\alpha^+$ DC preferentially present cell-associated or soluble blood-borne Ags to CD8$^+$ T cells (13, 14). Adoptive transfer studies indicate that CD8$^-$ DC favor induction of Th2-like responses whereas CD8$^+$ DC favor Th1-like responses (12, 15). Therefore, any perturbation of DC development that impacts on the relative abundance or function of either DC subtype may have profound effects on immune homeostasis.

Alterations in myeloid lineage development in NOD mice (3, 4) could influence the development and function of myeloid-derived DC and contribute to diabetes development. To address this issue, we examined the development of myeloid DC from bone marrow (BM) progenitors in vitro and DC populations in vivo in autoimmune diabetes-prone NOD mice, compared with recombinant congenic nonobese diabetes-resistant (NOR) mice and unrelated BALB/c and C57BL/6 (BL/6) strains.

## Materials and Methods

### *Mice*

Female NOD (NOD Lt/Jax, H-2$^{g7}$), NOR (NOR Lt, H-2$^{g7}$), NOD.scid (H-2$^{g7}$), BALB/c (H-2$^d$), CB17.scid (H-2$^d$), C57BL/6 (H-2$^b$), and CBA (H-2$^k$) mice were obtained from the Walter and Eliza Hall Institute central breeding facilities (Parkville, Victoria, Australia) and used at 6–10 wk of age. All studies were performed within institutional animal care guidelines.

### *Culture medium and reagents*

Culture medium (R-10) was RPMI 1640 (Life Technologies, Rockville, MD) with $10^{-3}$ M sodium pyruvate, $10^{-4}$ M nonessential amino acids (Life Technologies), $2 \times 10^{-3}$ M glutamine, $5 \times 10^{-5}$ M 2-ME (Sigma-Aldrich, St. Louis, MO), and 10% v/v FCS (JRH Biosciences, Lenexa,

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[3] Abbreviations used in this paper: T1D, type 1 diabetes; DC, dendritic cell; flt3-L, flt3 ligand; NOD, nonobese diabetic; MFI, mean fluorescence intensity; NOR, nonobese diabetes-resistant; BM, bone marrow; SCF, stem cell factor; rm, recombinant murine; CM, conditioned medium; HPC, hematopoietic progenitor cell; SCA, stem cell Ag.



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KS). Recombinant murine (rm)GM-CSF, rmIL-4, rmSCF, and rmTNF-$\alpha$ were from PeproTech (Rocky Hill, NJ). Recombinant human flt3-L was provided by Immunex (Seattle, WA). FITC-dextran (40,000 m.w.) and LPS (serotype 111:B4) were from Sigma-Aldrich.

### *Abs and flow cytometry*

Abs directed against CD3 (KT3), B220 (RA3-6B2), Ly-6G (Gr-1; RB6-8C5), Ly-76 (TER-119), F4/80 (F4/80), CD11b (M1/70), CD11c (N418), MHC class II (OX-6 (directed against a nonpolymorphic determinant on rat Ia, but also I-$A^{g7}$), 10.2.16 (I-$A^{k,g7,r,f,s}$), and M5/114 (I-$A^{b,d,q}$, I-$E^{d,k}$)), DEC-205 (NLDC-145), M-CSF R (AFS-98), and c-*kit* (ACK-2) were purified from hybridoma supernatants and used as purified mAb or conjugated in-house. Anti-CD8$\alpha$-FITC (CT-CD8a) was from Caltag Laboratories (Burlingame, CA). Other mAb directed to CD54 (3E2), IL-7R$\alpha$ (B12-1), CD40 (3/23), I-$A^b$ (AF6-120.1), I-$A^d$/I-$E^d$ (2G9), CD43 (S7), MAC-3 (M3/84), CD80 (16-10A1), and CD86 (GI-1) were purchased from BD PharMingen (San Diego, CA). Cells to be stained were preblocked by incubation in blocking mix (10% v/v normal mouse serum and 10% v/v of anti-CD16/32 (2.4G2) tissue culture supernatant) at 4°C for 5 min. Cells were incubated with FITC-, PE-, or biotin-conjugated primary Abs at 4°C for 30 min in blocking mix, washed in PBS/1.5% FCS, and incubated with streptavidin-FITC, streptavidin-PE, or streptavidin-Tricolor (Caltag Laboratories) as necessary before a final wash. Samples were analyzed on a FACScan (BD Biosciences, Mountain View, CA). Live cells were selected by forward/side scatter gating and/or propidium iodide exclusion. In most instances $10^4$ live-gated cells were collected for analysis. For analysis of BM, spleen, and thymus, $5 \times 10^4$, $5 \times 10^5$, or $10^6$ live events, respectively, were collected. Staining intensities were expressed as arithmetic mean fluorescence intensity (MFI) calculated using CellQuest 3.1 (BD Biosciences).

### *Generation of DC in vitro*

To propagate BM-derived DC, mice were euthanized by $CO_2$ narcosis and femurs and tibiae were collected into cold mouse tonicity PBS. Cells were flushed from the marrow cavity with PBS/2.5% FCS and erythrocytes were lysed with distilled water. After washing in R-10, cells were plated in six-well culture plates (Nunc, Rochester, NY) at $6 \times 10^6$ cells/well in 3 ml R-10. Cultures were supplemented with GM-CSF and IL-4 (1 ng/ml each unless stated otherwise) and maintained in 5% $CO_2$ at 37°C. In general, cultures were established from BM pooled from three mice. Nonadherent cells were removed by gentle washing after 2 days and half the medium was replaced with fresh R-10 containing GM-CSF and IL-4. No significant differences were determined among strains in the number of adherent cells remaining after this selection procedure. At day 5, nonadherent cells were removed by gentle washing and used as bulk DC or further enriched by centrifugation ($600 \times g$ for 10 min at room temperature) over a 14.5% metrizamide column (Sigma-Aldrich). Enrichment generally resulted in >95% $CD11c^+CD86^{high}$ cells.

When the effect of LPS on DC maturation was studied, LPS (1 $\mu$g/ml) was added for the final 18–20 h of culture. To determine the contribution of secreted mediators on DC development, conditioned medium (CM) was collected from day 5 GM-CSF/IL-4-supplemented BM cultures. Cells were removed by centrifugation and CM was stored at −20°C. Where noted, GM-CSF/IL-4-supplemented BM cultures were established using equal portions of CM and fresh R-10 supplemented with GM-CSF/IL-4. Nonadherent cells were removed as described and cultures were replenished to contain 50% CM. Bulk cells were harvested at day 5 and analyzed by flow cytometry. CM and R-10 (control) were added to BM cultures in all possible combinations. CM was derived from at least two independent sets of cultures, and the effect of CM addition was tested twice using pools of BM from three mice of each strain. DC generation from immunocompetent and lymphocyte-deficient SCID mice was compared in two experiments. Pooled BM (from three mice) was prepared from NOD, NOD.scid, BALB/c, or CB17.scid mice, and GM-CSF/IL-4-supplemented cultures were established as described. Bulk cells were harvested at day 5 and analyzed by flow cytometry.

Generation of DC in flt3-L-supplemented cultures was performed using a modification of the method described by Brasel et al. (16). Bulk BM cells were prepared as described above and plated in six-well plates at $6 \times 10^6$ cells/well in 3 ml R-10 supplemented with flt3-L (200 ng/ml). Cultures were maintained in 5% $CO_2$ at 37°C for 10 days and nonadherent cells were harvested by gentle washing.

For isolation of myeloid progenitors, erythrocyte-depleted BM was subjected to an initial round of immunomagnetic bead-mediated cell depletion using mAb directed at lineage markers. BM cells were incubated with mAb to CD3, B220, Ly-6G, CD11b, and Ly-76 at predetermined optimal concentrations (1–5 $\mu$g/ml) for 30 min at 4°C. Cells were washed (PBS/2.5% FCS) and mAb-coated cells were removed using anti-rat IgG Dynabeads (Dynal Biotech, Carlton South, Victoria, Australia) according to the manufacturer's instructions. Remaining cells were further incubated with biotin-conjugated mAb to lineage markers (CD3, B220, Ly-6G, CD11b, and Ly-76), IL-7R$\alpha$ (B12-1), and SCA-1 (E13-161.7), along with FITC-conjugated anti-c-*kit* for 30 min at 4°C. Cells were washed and incubated with streptavidin-PE for 30 min at 4°C and washed, propidium iodide was added (2 $\mu$g/ml), and FITC$^+$PE$^-$ (c-*kit*$^+$lineage$^-$IL-7R$\alpha^-$SCA-1$^-$) cells were obtained by sterile sorting using either FACStar (BD Biosciences) or MoFlo (Cytomation, Fort Collins, CO) instruments. Myeloid progenitors were added to 24-well plates at $10^4$ cells/well in 1 ml R-10 supplemented with GM-CSF/IL-4 (both 1 ng/ml) or GM-CSF/SCF/TNF-$\alpha$ (4, 20, and 10 ng/ml, respectively) and maintained at 37°C in 5% $CO_2$. Cultures were fed with 0.5 ml fresh R-10 containing the appropriate cytokines at day 4. Nonadherent cells were harvested for analysis at day 7.

### *Quantitation of endocytosis*

Nonadherent cells were harvested from GM-CSF/IL-4-supplemented BM cultures, washed, and equilibrated to 4°C or 37°C in R-10 ($10^6$ cells/500 $\mu$l); 0.2 volumes of FITC-dextran (5 mg/ml) was added, and cells were incubated for 2 h at the required temperature. Endocytosis was stopped by washing twice with ice-cold PBS containing 2.5% FCS and 0.02% sodium azide, and samples were maintained at 4°C for further steps. Controls were cells incubated at 4°C with or without FITC-dextran and cells incubated at 37°C without FITC-dextran. Cells were then stained as described with anti-CD86-PE and anti-CD11c-biotin, washed, and incubated with streptavidin-Tricolor (Caltag Laboratories) for 30 min on ice. After washing, cells were analyzed on a FACScan (BD Biosciences). Mature and immature DC populations were gated on the basis of CD11c and CD86 expression and the proportion of endocytically active cells calculated by subtracting background (cells incubated with FITC-dextran at 4°C) for each of the defined populations.

### *[$^3$H]Thymidine incorporation in BM cultures*

BM suspensions were prepared as described and added in triplicate to 96-well flat-bottom plates at $6 \times 10^5$ cells/well in 300 $\mu$l R-10. Cultures were supplemented with GM-CSF (1 ng/ml) and IL-4 (1 ng/ml) and maintained in 5% $CO_2$ at 37°C. At day 2, nonadherent cells were removed and cultures were replenished with 300 $\mu$l fresh R-10 containing GM-CSF (1 ng/ml) and IL-4 (1 ng/ml). [$^3$H]Thymidine (1 $\mu$Ci) was added for the respective 24-h periods. Cells were harvested onto glass filter mats and proliferation was assessed by [$^3$H]thymidine incorporation measured with a scintillation counter (Topcount; Packard, Groningen, The Netherlands). Results were expressed as gross cpm ± SD.

### *Preparation of single-cell suspensions of lymphoid tissues*

Spleens or thymi were excised and placed in ice-cold PBS. Individual organs were disrupted by gentle crushing with the end of a syringe plunger and suspended in 5 ml RPMI 1640/2% FCS containing collagenase (1 mg/ml, type III; Worthington, Freehold, NJ) and DNase I (1000 U/ml; Roche Diagnostics, Indianapolis, IN). Suspensions were maintained at room temperature for 25 min and intermittently resuspended by passage through a transfer pipette. EDTA (0.1 volumes of 100 mM in PBS) was added and pipetting was maintained continuously for another 5 min. Any undigested stromal material that remained was removed by passage through stainless steel mesh and an equal volume of ice-cold R-10 was added. From this point, cell suspensions were maintained at 4°C. Cells were collected by centrifugation and washed (R-10), and erythrocytes were lysed (spleen only; $NH_4Cl$/Tris buffer), washed, and finally resuspended in R-10.

### *Preparation of peripheral blood leukocytes and BM for flow cytometry*

Single-cell suspensions of BM were prepared as described above and erythrocytes were removed by lysis. For analysis of peripheral blood leukocytes, blood was obtained by cardiac puncture using heparinized syringes and needles and collected into Alsever's anticoagulant. Erythrocytes were removed by two rounds of lysis, cells were washed (PBS/2.5% FCS), and peripheral blood leukocytes were collected by centrifugation.

### *Cytospins*

Cytospins were prepared in a Cytospin 3 (Thermo Shandon, Pittsburgh, PA). Preparations were air-dried overnight and stained using Diff-Quik (Lab Aids, Narrabeen, Australia) for morphological analysis.

*Mixed leukocyte reactions*

MLRs were established using nylon wool-enriched splenic T cells ($2 \times 10^5$/well) and graded doses of gamma-irradiated (2000 rad, $^{60}$Co source) stimulator cells in R-10 (200 $\mu$l final volume in 96-well round-bottom plates). Syngeneic and allogeneic bulk spleen cells were routinely included as controls. MLR were maintained for 3 days and [$^3$H]thymidine (1 $\mu$Ci/well) was added during the final 18 h of culture. [$^3$H]Thymidine incorporation was assessed as above.

*Statistical analysis*

Group data were compared by ANOVA followed by Newman-Keuls post-test. Where indicated, Student's $t$ test was used for comparison of means. Flow cytometric data were corrected for non-normality by log transformation as required before analysis.

## Results

*Increased yield of CD11c$^+$ DC from GM-CSF/IL-4-supplemented NOD mouse BM cultures*

We first established conditions for comparing DC generation among strains that minimized manipulation of progenitor populations. Erythrocyte-lysed BM was cultured in the presence of GM-CSF/IL-4 for 2 days followed by removal of nonadherent cells. DC subsequently generated from the remaining adherent myeloid progenitors, as described by Inaba et al. (17), were harvested after another 3 days. In keeping with the findings of others (18, 19), IL-4 was required for efficient generation of mature DC (data not shown). With GM-CSF held at 1 ng/ml, little effect of IL-4 was observed at concentrations of 0.1 ng/ml or less. However, IL-4 at 1 ng/ml markedly reduced the proportion of Gr-1$^+$ myeloid progenitors/granulocytes and resulted in an increase in the proportion of CD11c$^+$ DC, particularly in NOD and NOR cultures. Further increasing the IL-4 concentration 10-fold to 10 ng/ml slightly reduced the scale of differences among strains, but CD11c$^+$ DC number remained highest in NOD cultures. As the GM-CSF concentration may be a key determinant of the relative number of DC generated across strains, we titrated the addition of GM-CSF in the presence of IL-4 (1 ng/ml). The combination of 1 ng/ml GM-CSF and 1 ng/ml IL-4 resulted in the highest percentage of CD11c$^+$ DC (Fig. 1*A*) and proportion of mature (CD11c$^+$CD86$^{high}$) DC (Fig. 1*B*) in each strain. As expected, the total number of DC was increased by higher GM-CSF concentrations, but this also increased the outgrowth of other cell types such as granulocytes and immature myeloid cells and favored proliferation at the expense of differentiation/maturation. Importantly, the relative yield of CD11c$^+$ DC in the presence of IL-4 was similar across strains over a wide range of GM-CSF concentrations (Fig. 1*C*). We next compared a range of GM-CSF/IL-4 combinations that encompassed those commonly used by various investigators (19–23). At all combinations tested DC generation from NOD exceeded that of other strains (Fig. 1*D*). We then used a combination of GM-CSF and IL-4 at 1 ng/ml each, the combination giving the highest percentage of CD11c$^+$ DC and mature DC while still producing fully functional DC. This combination avoided excessive outgrowth of non-DC myeloid cells and potential artifacts introduced by high cytokine concentrations. In cultures supplemented with 1 ng/ml GM-CSF and 1 ng/ml IL-4 the yield of nonadherent cells, which were mainly CD11c$^+$ DC, immature myeloid cells, and occasional granulocytes, did not differ significantly among strains at day 5 (Table I). The total number of CD11c$^+$ DC recovered was significantly greater from NOD than from NOR, BALB, or BL/6 cultures. The proportion of CD11c$^+$ DC was greatest in NOD and NOR cultures, exceeding that in BALB/c and BL/6 cultures by ~50%, indicating that, despite differing yields of DC, myeloid progenitors of both NOD and NOR mice shared an increased commitment to terminal differentiation to DC. Very few T or B lymphocytes were present in day 5 GM-CSF/IL-4-supplemented BM cultures. Moderately adherent cells that could be removed by vigorous washing with cold PBS/10 mM EDTA were almost exclusively CD11c$^+$CD86$^{-/low}$ immature DC (data not shown). Adherent macrophages, readily discernible by their extensively flattened morphology, comprised only a minor proportion of cells.

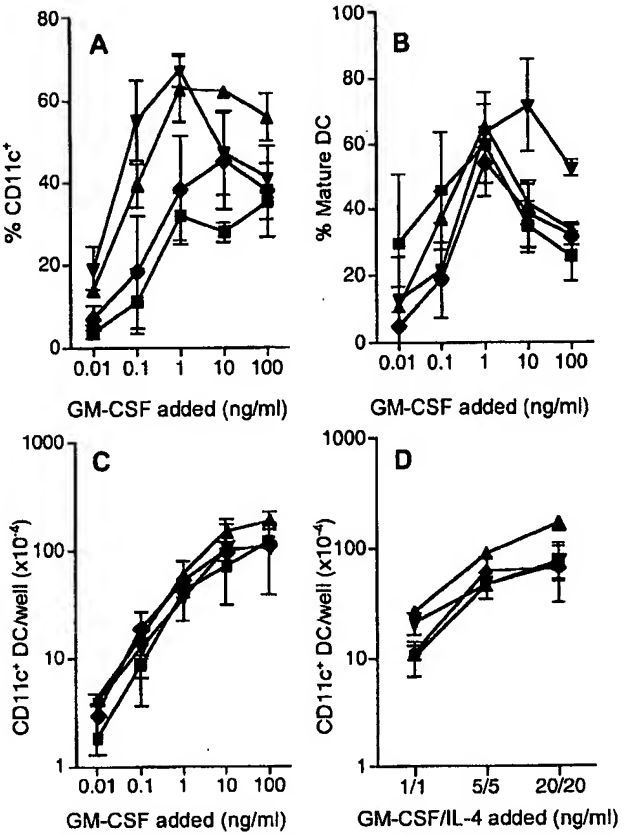



**FIGURE 1.** Optimization of GM-CSF/IL-4-supplemented BM culture conditions. *A*, Proportion of CD11c$^+$ DC in cultures at day 5. *B*, Proportion of CD11c$^+$ DC expressing the CD11c$^+$CD86$^{high}$ phenotype. *C* and *D*, Recovery of CD11c$^+$ DC from cultures at day 5. *A–C*, Cultures were established as described using NOD (▲), NOR (▼), BALB/c (◆), or C57BL/6 (■) BM in the presence of IL-4 (1 ng/ml) and graded concentrations of GM-CSF. Data are mean ± SD of two experiments, each with BM pooled from three mice. *D*, Cultures were established using NOD (▲), NOR (▼), BALB/c (◆), or C57BL/6 (■) BM in the presence of GM-CSF/IL-4 at the combinations shown; data are means from three animals in a single experiment. Results for NOD mice are significantly greater than for NOR, BALB/c, and C57BL/6 mice at each GM-CSF/IL-4 combination ($p < 0.05$, $p < 0.001$, and $p < 0.01$, respectively, at 1/1; $p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively, at 5/5; $p < 0.01$, $p < 0.01$, and $p < 0.01$, respectively, at 20/20).

*Increased costimulatory and adhesion molecule expression by mature NOD and NOR DC in GM-CSF/IL-4-supplemented BM cultures*

In GM-CSF/IL-4-supplemented BM cultures we found mixed populations of both phenotypically mature and immature DC bearing high or low levels, respectively, of the costimulatory molecule CD86 (Fig. 2), as reported by others (18–20). We used anti-CD11c to define DC for quantitative analysis due to the lack of anti-MHC class II mAb that react across the MHC haplotypes of all strains tested. The proportion of DC that exhibited the mature CD11c$^+$CD86$^{high}$ phenotype was greatest in BL/6 cultures but did not differ significantly among other strains (NOD, 35.1 ± 12.6

Table I. *Cell recovery from GM-CSF/IL-4-supplemented BM cultures*[a]

| | NOD | NOR | BALB/c | C57BL/6 |
|---|---|---|---|---|
| Cell recovery (% of starting BM) | 13.2 ± 4.8 (19) | 9.8 ± 2.2 (13) | 12.8 ± 4.5 (13) | 12.1 ± 3.5 (9) |
| Proportion $CD11c^+$ (%) | 46.3 ± 15.1 (18)[b] | 46.9 ± 16.0 (12)[b] | 32.9 ± 13.9 (15) | 25.0 ± 10.0 (11) |
| $CD11c^+$ DC yield ($10^4/10^6$ BM) | 6.3 ± 1.9 (15)[c] | 4.5 ± 0.8 (9) | 4.6 ± 1.6 (10) | 4.1 ± 1.3 (8) |

[a] Data presented are mean ± SD; the number of experiments appears in parentheses.
[b] Significantly greater than BALB/c ($p < 0.05$) and C57BL/6 ($p < 0.01$).
[c] Significantly greater than NOR, BALB/c ($p < 0.05$), and BL/6 ($p < 0.01$).

($n = 18$); NOR, 36.5 ± 14.6 (n = 12); BALB/c, 28 ± 14.2 ($n = 15$); BL/6, 59 ± 13.1 ($n = 8$); BL/6 significantly greater than NOD ($p < 0.05$) and BALB/c ($p < 0.001$). Mature DC could alternatively be identified as cells coexpressing high levels of MHC class II and CD86, whereas immature DC expressed only moderate levels of both (Fig. 2). Qualitative analysis indicated that MHC class II and CD86 coexpression defined a similar pattern of DC development across strains to that observed using CD11c and CD86. It is noteworthy that expression of MHC class II was not uniformly high on $CD86^{high}$ cells, particularly those from NOD and NOR cultures (Fig. 2). In addition to CD86 expression, mature and immature DC could also be distinguished on the basis of intermediate or high expression of CD11b, respectively. There was concordance across strains of $CD11b^{int}$ and $CD11b^{high}$ DC with $CD86^{high}$ and $CD86^{low}$ populations, respectively, which confirmed the findings based on CD86 discrimination of mature and immature populations.

While extending across an almost identical range in all strains, CD86 expression was consistently higher on mature and immature DC populations from NOD and NOR mice (Fig. 2, Table II). CD80 expression showed a similar trend (Table II), but the proportion of $CD11c^+CD80^{high}$ DC was similar across strains (29.2 ± 7.5, 32.3 ± 11, 26.1 ± 14, and 32.4 ± 20.3%; NOD, NOR, BALB/c, and BL/6, respectively). CD40 expression was elevated on NOD and NOR DC when relative levels within experiments were compared ($p < 0.001$), but not when MFI were pooled (Table II) before comparison. The adhesion molecule CD54 was expressed at moderate to high levels on DC from all strains, but a subpopulation of NOD DC had increased expression, resulting in a higher overall MFI (Table II). In contrast to the costimulatory molecules examined, CD11b expression was equivalent on respective mature or immature DC populations across strains. Expression of the myeloid/granulocyte- and macrophage-associated molecules Ly-6G (Gr-1) and F4/80 (average MFI, ~50 and 20, respectively) and CD43 (average MFI, ~50) was equivalent across strains, and DC from all strains were negative for MAC-3 expression. M-CSF receptor (c-*fms*) was expressed at equivalent levels on similar proportions (41–50%) of immature DC across strains. As expected, the proportion of mature DC that expressed M-CSF receptor was reduced but similar (18–30%) across strains. Thus, the phenotypic differences identified were selective and restricted to costimulatory and adhesion molecules.

### *DC from GM-CSF/IL-4-supplemented BM cultures correspond functionally across strains*

We sought to determine whether the altered pattern of DC development among strains was reflected in altered function. Endocytic activity, a marker of Ag uptake and processing by immature DC that is down-regulated upon maturation, was measured as FITC-dextran uptake. The majority of immature DC in each strain examined (70 ± 1, 67 ± 2, and 59 ± 1%, NOD, NOR, and BALB/c, respectively) were endocytically active. By comparison, activity was significantly reduced in mature DC (39 ± 2, 26 ± 7, and 24 ± 7.4%, respectively; all $p < 0.05$, $t$ test; $n = 3$). We also tested the ability of GM-CSF/IL-4-derived DC to respond to inflammatory stimuli by adding LPS during the final 20 h of culture. In response to LPS, CD86 and MHC class II expression was up-regulated on both mature and immature DC populations in NOD, NOR, BALB/c, and BL/6 cultures; furthermore, LPS increased the numbers of $CD11c^+CD86^{high}$ (or MHC class $II^{high}CD86^{high}$) DC in each strain (data not shown).

We next determined whether developmental differences among strains affected T cell stimulatory capacity. Mature DC were metrizamide-enriched from GM-CSF/IL-4-supplemented BM cultures and used in allogeneic and syngeneic MLR cultures to assess their ability to provide costimulatory signals. DC from NOD, BALB/c, and BL/6 elicited similar proliferative responses in parallel allogeneic MLR assays (Fig. 3*A*). Despite a small degree of interassay variability, no consistent differences were observed in the allostimulatory capacity of NOD and NOR DC (Fig. 3*B*). While it is difficult to make direct comparisons among strains due

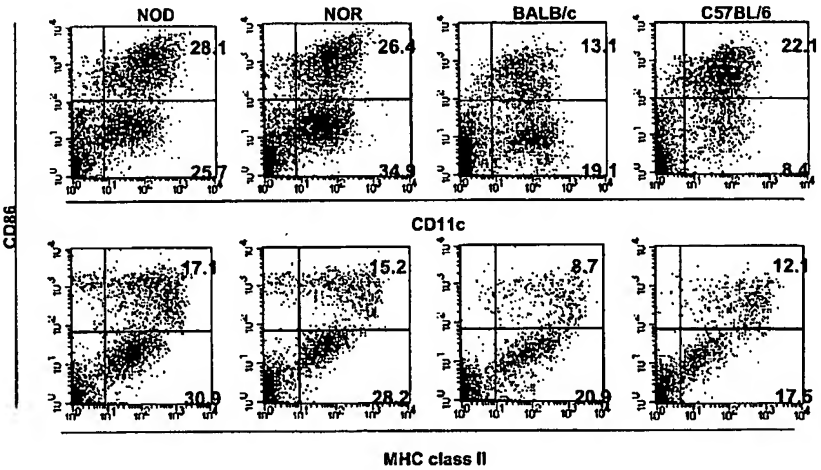



FIGURE 2. Bulk nonadherent cells from GM-CSF/IL-4-supplemented BM cultures contain phenotypically mature and immature $CD11c^+$ DC. Cells harvested at day 5 were stained with anti-CD11c and anti-CD86 or anti-MHC class II and anti-CD86. Cells were ungated other than for exclusion of dead cells based on propidium iodide uptake.

Table II. *Costimulatory and adhesion molecule expression on DC from GM-CSF/IL-4-supplemented BM cultures*[a]

| | CD86 | | CD80 | | | |
|---|---|---|---|---|---|---|
| | $CD11c^{+}$[b] | $11c^{+}86^{high}$[c] | $CD11c^{+}$[b] | $11c^{+}80^{high}$[c] | CD40[b] | CD54[b] |
| NOD | 342 ± 192[d] | 968 ± 475[e] | 132 ± 29[f] | 343 ± 128[d] | 45 ± 31 | 415 ± 147[g] |
| NOR | 433 ± 252[d] | 906 ± 379[e] | 122 ± 22[f] | 269 ± 72 | 50 ± 31 | 267 ± 54 |
| BALB/c | 197 ± 120 | 567 ± 235 | 82 ± 26 | 246 ± 70 | 21 ± 14 | 219 ± 82 |
| C57BL/6 | 174 ± 118 | 444 ± 177 | 74 ± 23 | 189 ± 58 | 18 ± 10 | 228 ± 118 |

[a] Data are mean ± SD MFI from 10–14 experiments depending on strain.
[b] MFI of $CD11c^{+}$-gated population.
[c] MFI of $CD11c^{+}$ $CD86^{high}$ or $CD11c^{+}$ $CD80^{high}$-gated population as noted.
[d] Significantly greater than BALB/c, BL/6 ($p < 0.05$).
[e] Significantly greater than BALB/c ($p < 0.01$), BL/6 ($p < 0.001$).
[f] Significantly greater than BALB/c ($p < 0.05$).
[g] Significantly greater than NOR ($p < 0.01$), BALB/c ($p < 0.001$), BL/6 ($p < 0.01$).

to MHC disparities, NOD DC stimulated similar levels of proliferation in syngeneic MLR cultures compared with NOR, BALB/c, and BL/6 DC. This was particularly evident when bulk allogeneic spleen cells were used as internal controls (Fig. 4). No consistent differences were observed between the stimulatory capacity of MHC-identical NOD- or NOR-derived DC when examined in crossover syngeneic MLR cultures (Fig. 4, *A* and *B*).

*Increased proliferation in GM-CSF/IL-4-supplemented NOD BM cultures*

While increased commitment to DC generation was observed in both NOD and NOR BM cultures, the number of DC generated was higher only in NOD. We set out to determine whether increased myeloid progenitor proliferation contributed to the greater yield of NOD DC. As lymphocytes and other nonmyeloid cells are removed from cultures in the nonadherent cell fraction at day 2 (17), proliferation of the remaining myeloid progenitor pool could be assessed by [$^3$H]thymidine incorporation. Proliferation was greatest in the 2 days following nonadherent cell removal (Table III) and, notably, in the initial 24 h was significantly greater in NOD than in NOR and BALB/c cultures. DC are terminally differentiated and develop progressively 2–3 days after myeloid progenitor division (17). Therefore, our results are consistent with increased proliferation of myeloid progenitors in NOD cultures around day 2–3, leading to an increased number of DC generated by day 5 in NOD relative to NOR cultures.

*The pattern of myeloid DC development reflects myeloid progenitor programming*

The differences in the yield of DC among strains could reflect differences in the relative number of myeloid progenitors or their

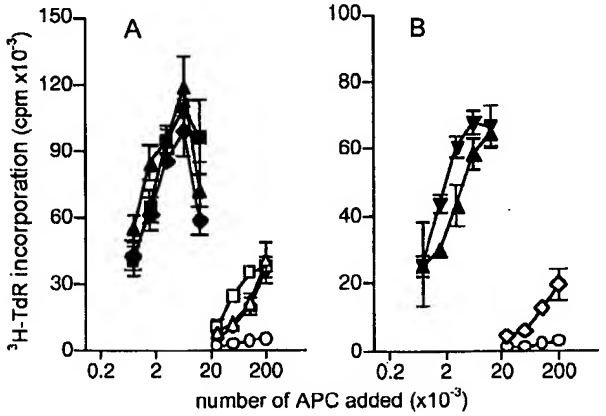



**FIGURE 3.** Allostimulatory activity of mature BM-derived DC. DC were propagated in GM-CSF and IL-4 and purified by density separation over metrizamide columns. Allogeneic MLR were established with 2 × $10^5$ nylon wool-passaged CBA T cells and graded doses of DC or bulk splenocytes as stimulators. Stimulators in *A* were DC from NOD (▲), BALB/c (◆), and C57BL/6 (■), or splenocytes from NOD (△), BALB/c (◇), C57BL/6 (□), and CBA (○) mice; stimulators in *B* were DC from NOD (▲) and NOR (▼) or splenocytes from BALB/c (◇) and CBA (○) mice. Results are representative of at least six experiments.

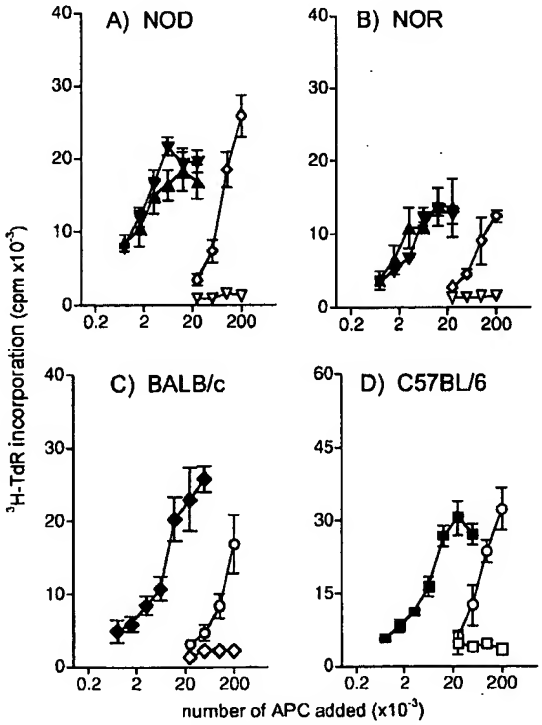



**FIGURE 4.** Ability of mature BM-derived DC to stimulate syngeneic MLR assays. DC were propagated in GM-CSF and IL-4 and purified by density separation over metrizamide columns. Syngeneic MLR were established with 2 × $10^5$ nylon wool-passaged T cells and graded doses of DC or bulk splenocytes as stimulators. *A*, NOD T cells stimulated with NOD DC (▲), NOR DC (▼), allogeneic BALB/c (◇), or syngeneic NOD (▽) splenocytes. *B*, NOR T cells stimulated with NOD DC (▲), NOR DC (▼), allogeneic BALB/c (◇), or syngeneic NOR (▽) splenocytes. *C*, BALB/c T cells stimulated with BALB/c DC (◆), allogeneic CBA (○), or syngeneic (BALB/c) (◇) splenocytes. *D*, C57BL/6 T cells stimulated with C57BL/6 DC (■), allogeneic CBA (○), or syngeneic C57BL/6 (□) splenocytes. Results are representative of four to six experiments, depending on strain.

Table III. *Proliferation in GM-CSF/IL-4-supplemented BM cultures*[a]

| Culture Period (days) | [$^3$H]TdR Incorporation (cpm × $10^{-3}$) | | |
|---|---|---|---|
| | NOD | NOR | BALB/c |
| 2–3 | 115 ± 28[b] | 74 ± 18 | 59 ± 19 |
| 3–4 | 104 ± 59 | 79 ± 39 | 74 ± 23 |
| 4–5 | 57 ± 42 | 63 ± 42 | 63 ± 41 |

[a] Data are from four (NOD and NOR) or three (BALB/c) experiments in which cultures were performed in parallel.
[b] Significantly greater than NOR and BALB/c ($p < 0.05$).

adhesive properties; therefore, we investigated whether the pattern of DC development observed in bulk BM cultures was determined at the level of myeloid-committed progenitors. The recent description of early myeloid progenitors (24) as lineage$^-$IL-7R$\alpha^-$SCA-1$^-$c-*kit*$^+$ cells allowed us to compare DC development from these cells. BALB/c mice were not analyzed, as they carry the Ly6.1 allotype and do not express SCA-1 (Ly6A-E) on hematopoietic progenitors. Myeloid progenitors obtained by cell sorting were cultured in GM-CSF/IL-4 or GM-CSF/SCF/TNF-$\alpha$, a second cytokine combination commonly used to generate myeloid DC in vitro. Expansion of cell number (70- to 150-fold) was greatest from NOD myeloid progenitors (Table IV). Likewise, the number of CD11c$^+$ DC generated from NOD myeloid progenitors was ~1.5- to 2-fold that from NOR or BL/6 (Table IV). In GM-CSF/IL-4-supplemented myeloid progenitor cultures, the proportion of mature CD86$^{high}$ DC was greater from BL/6 than from NOD and NOR, mirroring the pattern seen in bulk BM cultures. Only a minority of DC expressed a mature phenotype in GM-CSF/SCF/TNF-$\alpha$-supplemented cultures, precluding analysis of CD86 expression. Thus, under two different cytokine combinations that promote myeloid DC development, phenotypically defined and purified myeloid-committed progenitors from NOD BM gave rise to more DC relative not only to unrelated BL/6 but also to closely related NOR mice.

To determine whether the pattern of DC maturation observed in bulk BM cultures may have been influenced by soluble factors secreted into the medium, CM was harvested from day 5 cultures and added to freshly prepared GM-CSF/IL-4-supplemented BM cultures. CM in the culture did not alter the pattern of DC development in any combination, regardless of the strain from which CM or BM was prepared (data not shown). In addition, by comparing GM-CSF/IL-4-supplemented BM cultures from NOD.scid and CB.17 SCID mice with cultures from control immunocompetent NOD and BALB/c mice, we determined that lymphocytes present in BM did not contribute to the development patterns observed (data not shown). These finding provide further evidence that the pattern of DC development was determined in a cell-intrinsic manner.

### *Increased yield of DC in flt3-L-supplemented NOD and NOR BM cultures*

To determine whether the altered expansion potential observed for NOD myeloid progenitors extended to early uncommitted progenitors cells we generated DC using the early-acting hematopoietic growth factor flt3-L. In flt3-L-supplemented BM cultures, DC develop as either CD11c$^+$CD11b$^+$ or CD11c$^+$CD11b$^-$ populations (Fig. 5), proposed to correspond to CD8$\alpha^-$ (myeloid) DC and CD8$\alpha^+$ (lymphoid-related) DC, respectively (16). Nonadherent cell recovery from day 10 flt3-L-supplemented BM cultures was significantly greater ($p < 0.01$) from NOD and NOR compared with BALB/c and BL/6 (50 ± 12 and 49 ± 13% vs 23 ± 10 and 23 ± 7%, respectively; $n = 4$ experiments, all strains tested in parallel). As nonadherent cells recovered were primarily CD11c$^+$ DC (range 80–95%), the DC yield was closely related to overall cell recovery, and the total number of CD11c$^+$ cells recovered was consistently greater from NOD and NOR (Fig. 5). Furthermore, recovery of DC with the CD11c$^+$CD11b$^+$ myeloid phenotype from flt3-L-supplemented BM was greatest from NOD and NOR cultures. As the proportion of lineage$^-$flt3$^+$ cells in BM was similar across strains (<1%) the increased recovery of DC from NOD and NOR cultures reflected greater expansion and DC development from flt3-L-responsive progenitor cells in these strains.

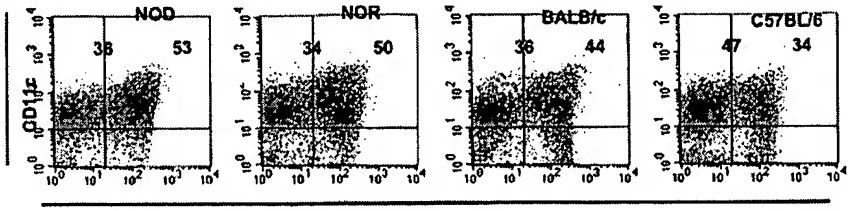
### *In NOD and NOR mice, DC are skewed toward the myeloid CD11b$^+$ subset in spleen and increased in blood and thymus*

To determine whether the increased proliferative capacity and number of DC precursors in NOD BM were mirrored in vivo we first examined the frequency of DC in peripheral blood leukocytes. CD11c$^+$ cells, most likely comprising mature DC as well as DC precursors, were more abundant in peripheral blood leukocytes recovered from NOD and NOR mice (Table V). We then examined DC in thymus (10 wk old) and spleen (3 and 10 wk old) of NOD, NOR, BALB/c, and BL/6 mice. Single-cell suspensions freshly prepared from individual animals were analyzed to obtain a representative estimate and avoid the nonspecific cell losses and potential artifacts associated with extensive cell depletion and/or enrichment steps commonly used in DC characterization. Thymic DC were readily distinguished as a CD11c$^{high}$ population (Fig. 6), and expression of CD11c and CD8$\alpha$ was consistent with that reported (6, 25). NOD and NOR thymi contained a significantly

Table IV. *Generation of DC from myeloid progenitors*[a]

| | NOD | | NOR | | C57BL/6 | |
|---|---|---|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 |
| GM-CSF + IL-4 | | | | | | |
| Cell recovery (× $10^{-4}$)[b] | 97.5 | 79.4 | 72.0 | 46.6 | 40.5 | 31.7 |
| Total CD11c$^+$ (× $10^{-4}$) | 75.1 | 37.8 | 47.8 | 19.9 | 33.2 | 18.2 |
| Proportion CD86$^{high}$ (%)[c] | 11.1 | 18.1 | 12.3 | 26.3 | 40.5 | 43.2 |
| CD86 (MFI)[d] | 719 | 802 | 549 | 520 | 530 | 438 |
| GM-CSF + SCF + TNF-$\alpha$ | | | | | | |
| Cell recovery (× $10^{-4}$)[b] | 159.0 | 70.0 | 127.5 | 73.6 | 67.5 | 30.3 |
| Total CD11c$^+$ (× $10^{-4}$) | 80.1 | 39.9 | 35.7 | 26.0 | 30.2 | 16.0 |
| Proportion CD86$^{high}$ (%)[c] | 4.4 | 4.9 | 3.8 | 5.2 | 3.6 | 8.1 |

[a] Data presented are from two separate experiments.
[b] Cultures were established as described in *Materials and Methods* and yield of total and CD11c$^+$ cells was determined.
[c] Proportion of all CD11c$^+$.
[d] CD86 MFI on CD11c$^+$CD86$^{high}$ population.




| | NOD | NOR | BALB/c | C57BL/6 |
|---|---|---|---|---|
| CD11c⁺ * ($10^4$/$10^6$ BM) | 49.8 ± 11.8† | 43.4 ± 9.7† | 20.1 ± 7.7 | 21.8 ± 5.0 |
| Ratio 11c⁺/11b⁺: 11c⁺/11b⁻ | 1.01 ± 0.37 | 0.98 ± 0.45 | 1.13 ± 0.36 | 0.68 ± 0.20 |
| CD11c⁺/CD11b⁺ * ($10^4$/$10^6$ BM) | 24.3 ± 7.2‡ | 21.0 ± 9.1‡ | 10.3 ± 3.9 | 8.2 ± 2.1 |

**FIGURE 5.** Generation of BM-derived DC in cultures supplemented with flt3-L. BM cultures were established in the presence of flt3-L as described in *Materials and Methods*. *Upper panel*, Nonadherent cells were harvested at day 10 and stained with anti-CD11c and anti-CD11b. Shown are percentages of cells lying in quadrants containing $CD11c^+$ $CD11b^-$ and $CD11c^-$ $CD11b^+$ cells. Dot plots are from a single experiment representative of four independent experiments in which all strains were examined in parallel. *Lower panel*, Data pooled from these four experiments. *, Total recovery of each phenotypically defined cell subset ($10^4$ cells per $10^6$ BM cells used to establish cultures); †, significantly greater than BALB/c, BL/6 ($p < 0.01$); ‡, significantly greater than BALB/c and BL/6 ($p < 0.05$).

greater proportion of $CD11c^+$ DC (Table V). This was not due to a relative enrichment of DC, as total cellularity did not differ significantly across strains. To determine whether the increased generation of DC from NOD and NOR progenitors influenced the normally dominant $CD8\alpha^+$ DC subtype in the thymus, $CD8\alpha$ and CD11b expression was also examined. In all strains, the majority (65–71%) of thymic DC expressed $CD8\alpha$ (Fig. 6) and the proportion expressing either high or intermediate/low levels of $CD8\alpha$ did not differ. In all strains, CD11b was present on only a minority of thymic DC (12–17%). Therefore, while the relative abundance of DC in the thymus varied among strains, the balance of phenotypically defined DC subtypes did not.

Splenic DC were also readily distinguished as a $CD11c^{high}$ population, and DC subtypes were clearly defined by the presence or absence of $CD8\alpha$ or CD11b expression (Fig. 6). At 3 but not 10 wk of age, splenic DC were more abundant in NOD mice (Table V). At both ages, the $CD11c^+CD11b^+$ (or the corresponding $CD11c^+CD8\alpha^-$) subtype predominated, and was most abundant in NOD mice (Table VI).

## Discussion

Based on the premise that altered development of myeloid-derived DC could contribute to the pathogenesis of T1D, we examined development of DC in vitro from the autoimmune-prone, spontaneously diabetic NOD mouse, the related non-diabetes-prone NOR mouse, and unrelated non-diabetes-prone strains. In GM-CSF/IL-4-supplemented conditions, DC commitment and expression of costimulatory and adhesion molecules on DC was greater in NOD and NOR mice relative to unrelated strains. However, DC were generated in greater numbers from NOD compared with NOR mice due to increased proliferation and expansion of NOD myeloid progenitors. Our findings of increased expansion potential of NOD myeloid progenitors are consistent with previous observations of increased cycling of stem cells and greater numbers of GM-CFC in NOD BM (26). In contrast, two recent studies using GM-CSF in the absence of IL-4 concluded that DC development from NOD BM is impaired (27, 28). When we used the combination of GM-CSF and IL-4, superior for generating mature DC (18, 19, 21, 29), we found that DC development from NOD myeloid progenitors was increased rather than reduced. Another report suggesting that the yield of DC from GM-CSF/IL-4-supplemented NOD BM cultures is reduced (22) enumerated only mature (low buoyant density) DC separated on density gradients, thereby excluding the large number of less buoyant immature DC we also enumerate. Additionally, our cultures were established with whole BM to avoid manipulating progenitor populations. Others have used complement-depleted BM (22, 27), which could inadvertently alter the relative proportions of progenitor populations. Significantly, the elevated expression of CD86, CD80, and CD40 we observed is consistent with previous reports of GM-CSF/IL-4-generated NOD BMDC (22, 30). While DC from NOD and other strains were functionally similar in the assays we used, this does not preclude other as yet undefined functional differences.

The exact nature of the cell type(s) that give rise to DC in flt3-L-supplemented BM cultures is yet to be determined. Loss of flt3 expression from hematopoietic progenitor cells (HPC) as differentiation proceeds (reviewed in Ref. 31) and generation of DC from $flt3^+$ cells (16) suggest that flt3-L drives DC development from early HPC. Consistent with this, lineage-committed lymphoid or myeloid progenitors retain some responsiveness to flt3-L,

Table V. *Frequency of $CD11c^+$ DC*

| | Relative Frequency of $CD11c^+$ DC (%) | | | |
|---|---|---|---|---|
| | NOD | NOR | BALB/c | C57BL/6 |
| Blood ($n$ = 3) | 3.8 ± 0.3[a] | 4.7 ± 0.7[b] | 2.5 ± 0.4 | 2.7 ± 0.5 |
| Thymus ($n$ = 6) | 0.89 ± 0.11[c] | 0.91 ± 0.08[c] | 0.42 ± 0.10 | 0.60 ± 0.12[d] |
| Spleen | | | | |
| 3 wk ($n$ = 6) | 3.2 ± 0.8[e] | 2.4 ± 0.3 | 1.7 ± 0.2 | 1.7 ± 0.6 |
| 10 wk ($n$ = 3) | 3.2 ± 0.6 | 3.3 ± 0.4 | 2.7 ± 0.5 | 3.5 ± 0.2 |

[a] Significantly greater than BALB/c and BL/6 ($p < 0.05$).
[b] Significantly greater than BALB/c and BL/6 ($p < 0.01$).
[c] Significantly greater than BALB/c and BL/6 ($p < 0.001$).
[d] Significantly greater than BALB/c ($p < 0.01$).
[e] Significantly greater than NOR ($p < 0.05$), BALB/c, and BL/6 ($p < 0.01$).

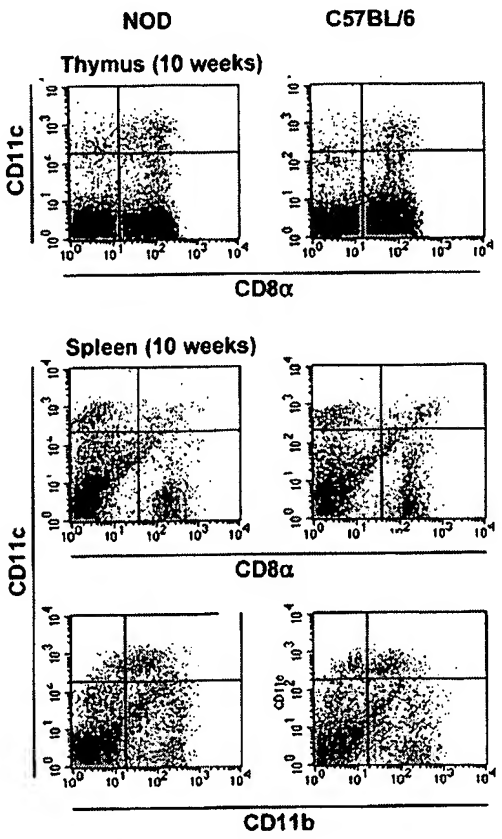



**FIGURE 6.** Phenotypic analysis of DC in thymus and spleen. Single-cell suspensions of thymus or spleen were stained for CD11c and CD8α or CD11b and analyzed by flow cytometry. Shown are dot plots from NOD or C57BL/6 mice gated for live cells by propidium iodide exclusion.

but DC development from these cells in vitro depends primarily on other growth factors (IL-7 and GM-CSF, respectively) (32, 33). Our findings show that early uncommitted HPC from both NOD and NOR share an increased expansion and DC-generative capacity in response to flt3-L relative to unrelated strains. In contrast, myeloid-committed progenitors in BM exhibit increased expansion capacity only in NOD mice. The exact mechanism underlying the increased proliferation of myeloid progenitors in NOD mice is unclear but could be either dysregulation of cell cycle control or enhanced cytokine responsiveness.

Members of the NF-κB family are important regulators of DC development and maturation (34, 35). The presence of NF-κB binding sites in the promoter regions of CD86, CD80, and CD40 (36–38) suggests that increased activation and nuclear translocation of NF-κB molecules could contribute to the selective up-regulation of these molecules in NOD and NOR DC. Increased commitment to DC development within the myeloid lineage of NOD and NOR is also consistent with increased NF-κB activation. The dependence of myeloid DC on RelB activation and nuclear translocation for differentiation (35, 39), and in particular terminal activation (40), indicates it may be a key mediator of enhanced myeloid DC development in NOD mice. Alternatively, other transcription factors, such as early growth response 1, which enhances monocyte development at the expense of granulocyte and erythroid differentiation (41), could also be important. Altered kinetics of NF-κB activation could also underlie the differences in maturation between strains, because GM-CSF/IL-4-supplemented NOD BM cultures eventually become more enriched for mature DC if maintained until day 7. The suggestion that NF-κB may be important in altered development of NOD DC is supported by a recent report showing hyperactivation of NF-κB in NOD DC in response to LPS stimulation (23). The similarity of DC stimulatory capacity among strains was unexpected in light of increased costimulatory molecule expression on NOD and NOR DC; however, it is possible that following DC:T cell interaction (and NF-κB activation) little further up-regulation of costimulatory molecules occurs on NOD and NOR DC, whereas expression on BALB/c and BL/6 DC is up-regulated to levels equivalent to NOD and NOR.

We found that the populations of DC subtypes in vivo in NOD mice reflected the differences in DC development in vitro in GM-CSF/IL-4 and flt3-L. GM-CSF and flt3-L are both important mediators of DC development in vitro (16, 17, 32, 33) but have different effects in vivo. flt3-L mobilizes and expands HPC (42) and results in accumulation of DC of both proposed subtypes in mice and humans (43–46). Conversely, mice lacking flt3-L have a substantial reduction in both DC subtypes (47). In contrast, GM-CSF is redundant in regulating DC numbers (48) or myeloid cells in the steady state (49) but is required to sustain myeloid inflammatory responses (50). It expands myeloid DC in mice (12) and most likely regulates inflammatory site DC derived from monocytes (51). The increased DC frequency shared by NOD and NOR mice in vivo is consistent with increased DC generation from early flt3-responsive HPC, as suggested by the increased generation of DC in flt3-L-supplemented NOD and NOR cultures. We show that

Table VI. *Frequency of $CD11c^+CD11b^+$ and $CD11c^+CD8^-$ DC in spleen*[a]

| | Proportion (% of total $CD11c^+$) | | | |
|---|---|---|---|---|
| | NOD | NOR | BALB/c | C57BL/6 |
| 3 wk | | | | |
| $CD11c^+$ $CD11b^+$ | 68.2 ± 3.7[b] | 60.7 ± 3.6[c] | 62.3 ± 2.2[c] | 56.7 ± 2.2 |
| $CD11c^+CD8^-$ | 69.1 ± 1.6[d] | 63.6 ± 2.4 | 70.1 ± 3.0[e] | 64.9 ± 2.5 |
| 10 wk | | | | |
| $CD11c^+$ $CD11b^+$ | 77.3 ± 2.2[f] | 72.4 ± 1.0[g] | 69.5 ± 2.7 | 66.4 ± 0.9 |
| $CD11c^+$ $CD8^-$ | 75.2 ± 0.6[h] | 71.6 ± 3.1[i] | 67.6 ± 1.7 | 64.0 ± 2.3 |

[a] Data are mean ± SD; $n = 6$ for 3 wk; $n = 3$ for 10 wk.
[b] Significantly greater than NOR, BALB/c ($p < 0.01$), and BL6 ($p < 0.001$).
[c] Significantly greater than BL/6 ($p < 0.05$).
[d] Significantly greater than NOR ($p < 0.01$), and BL/6 ($p < 0.05$).
[e] Significantly greater than NOR and BL/6 ($p < 0.01$).
[f] Significantly greater than NOR ($p < 0.05$), BALB/c, and BL/6 ($p < 0.01$).
[g] Significantly greater than BL/6 ($p < 0.05$).
[h] Significantly greater than BALB/c ($p < 0.01$) and BL/6 ($p < 0.001$).
[i] Significantly greater than BL/6 ($p < 0.01$).

differential sensitivities to hematopoietic growth factors that drive the different DC development streams exist among strains. It is possible, in fact, that differential responsiveness to flt3-L and GM-CSF in vivo determines the differences observed in DC population subtypes among strains. Mobilization and expansion of DC in vivo by administration of flt3-L and/or GM-CSF is likely to reflect the patterns observed in vitro. Both myeloid- and lymphoid-committed progenitors have been shown to give rise to CD8$\alpha^-$ and CD8$\alpha^+$ DC subtypes following adoptive transfer (8, 9, 33). While the spleen is permissive for both CD8$\alpha^+$ and CD8$\alpha^-$ DC development, the latter subtype predominates. However, the relative contribution of the myeloid and lymphoid lineages to each DC development stream within the spleen under steady-state conditions is currently unclear. Despite these uncertainties, the increased proportion of CD8$\alpha^-$ (myeloid) DC in NOD spleen reflects the increased generation of myeloid-derived DC observed in vitro.

Our findings indicate that the NOD genetic background confers broad alterations in hemopoiesis and DC generation that can be detected as increased progenitor proliferation, commitment to DC terminal differentiation within the myeloid lineage, and increased expression of costimulatory molecules on mature DC. These alterations could predispose to autoimmunity in the NOD mouse. While MHC class II molecule I-A$^{g7}$ is the major susceptibility allele (*Idd1*) for T1D in NOD mice, at least 16 other susceptibility alleles also contribute (reviewed in Ref. 52). NOR mice are recombinant congenic NOD mice carrying portions (11–12%) of the C57BL/KsJ genome that impart resistance to pancreatic islet inflammation (insulitis) and protection from diabetes (53, 54), while retaining NOD-derived *Idd1*, *2*, *3*, *6*, *7*, *8*, *10*, *12*, and *14* alleles (55). We found that myeloid-committed progenitors of NOR mice lack the increased proliferative capacity characteristic of NOD mice. This suggests the latter trait may be an important element in the development of spontaneous diabetes in NOD mice, and that the locus controlling the latter trait may have been replaced in NOR mice. Further investigation of mice congenic for single *Idd* alleles may help identify the genetic loci determining these effects.

The alterations in DC development described in this work could have an impact on the development of T1D by, on the one hand promoting pathogenic immunity or, on the other, impairing protective immunity. The thymus preferentially supports development of CD8$\alpha^+$ DC, even from myeloid progenitors (9), which are estimated to contribute 50% of thymus DC in C57BL/Ka mice (33). From our findings it is plausible to extrapolate that myeloid progenitors make an even greater contribution to thymic DC development in NOD mice. If thymic DC derived from myeloid progenitors were impaired in their ability to mediate negative selection, this could lead to escape of high-affinity autoreactive T cells from NOD thymus. Infiltration of the islets in female NOD mice by macrophages and TNF-$\alpha$-producing DC precedes T cell infiltration (56, 57) and is an essential initial step in disease progression (58). The expanded pool of immediate DC precursors in BM and blood of NOD mice may be readily recruited to pancreatic islets in response to an as yet unknown environmental or developmental signal and precipitate disease by local production of TNF-$\alpha$ and/or transfer of Ag to regional lymphoid tissues. Conversely, reduced numbers of CD8$\alpha^+$ DC that cross-present Ag to CTL (13) and may be responsible for induction of cross-tolerance (59) could impair peripheral deletion of autoreactive CTL.

In summary, our findings demonstrate that NOD mice have increased development of DC from myeloid progenitors in vitro, reflected in the homeostasis of DC populations in vivo. This shift toward myeloid-derived DC could predispose to autoimmune disease.

## Acknowledgments

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# Hematopoietic stem cell therapy for type 1 diabetes: induction of tolerance and islet cell neogenesis

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**Abstract**

Diabetes is a chronic disease with significant morbidity and mortality. Pancreas or islet cell transplantation is limited by a shortage of donors and chronic immune suppression to prevent allograft rejection. Consequently, interest exists in islet cell neogenesis from embryonic or mesenchymal stem cell as a possible cure for diabetes. However, unless tolerance to islet cells is re-established, diabetes treated by islet cell transplantation would remain a chronic disease secondary to immune suppression related morbidity. If islet cell tolerance could be re-induced, a major clinical hurdle to curing diabetes by islet cell neogenesis may be overcome. Recent studies suggest that adult hematopoietic stem cells (HSC) can reintroduce tolerance to auto-antigens. It is possible that HSC may also be able to switch lineage and, therefore, be a convenient source of stem cells for both inducing tolerance and islet cell regeneration. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Stem cell therapy; Islet cell neogenesis; Type 1 diabetes

## 1. Type 1 diabetes: an autoimmune disease

Evidence that diabetes is an autoimmune disease comes from: animal models such as non-obese diabetic (NOD) mice [1], protective and susceptibility HLA associations [2], cytotoxic T cells and auto-antibodies against islet cell antigens such as glutamic acid decarboxylase (GAD) [3,4], rare case reports of disease transfer from donor to recipient following HSC transplant [5], and, response to immune suppression [6].

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## 2. Morbidity and mortality of diabetes

Insulin, while prolonging life, is not a cure. Morbidity and mortality is usually a result of infections or diabetic vasculopathy (Table 1). Vascular complications result from hypertension, hyperlipidemia, and advanced glycosylated end products (AGEs) [7]. AGEs arise from hyperglycemic-related post-translational glycosylation of intra and extra-cellular proteins. These glycosylated proteins are thought to increase free radical injury to endothelium resulting in accelerated atherosclerosis [8]. Clinically, this translates to correlation of disease-related mortality with mean blood glucose levels. In fact, for every 1% increase

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Table 1
Morbidity and mortality of diabetes

| | |
|---|---|
| 1 | 300–600% higher mortality than the general population |
| 2 | Sixth leading cause of death in the America |
| 3 | Most common cause for blindness in America |
| 4 | Most common cause for dialysis in America |
| 5 | Death under age 20 generally from hypoglycemia, ketoacidosis, or infection |
| 6 | Death above age 20 generally from renal and/or vascular disease |
| 7 | Age-adjusted incidence of myocardial infarction is 4–6 times higher, and survival following myocardial infarction is worse, compared to non-diabetics |
| 8 | Cerebrovascular accidents (CVA) are more common in diabetics |
| 9 | Peripheral vascular disease with claudification common |
| 10 | Minor trauma may precipitate gangrene, ulcer and lower extremity amputation |

in HgbA1c, mortality increases approximately 11% [9–12].

## 3. Insulin treatment for type 1 diabetes

Diabetes is treated with either conventional insulin therapy or intensive insulin therapy (IIT). Since the risk of cardiovascular complications correlates with level of hyperglycemia (HgbA1c), the goal of IIT is tight control of blood sugar. While IIT is known to slow disease (e.g. retinopathy, nephropathy) progression by 35–70% [10–12], it does not prevent eventual development of these complications [10–13].

IIT has a higher incidence of hypoglycemic reactions (more than three times higher than conventional insulin treatment) that may cause seizures and or death [14]. IIT requires meticulous monitoring of blood sugar (4–6 times a day), frequent insulin injections (more than 3 times per day or an insulin pump), close control of diet, and is practical only in highly motivated groups. As quoted from the literature 'Achieving optimal blood glucose control, without an unacceptable rate of hypoglycaemia or unacceptable restrictions on lifestyle, is not simple with presently available insulin preparations and monitoring tools' [14]. 'Accordingly the appropriate use of insulin to obtain good metabolic control requires the continued and informed expertise of both patient and advising professional, but also attention from both to self-motivation in order to make the desired lifestyle changes possible' [14]. The incremental cost per year of life gained by IIT has been estimated at US$28, 661 [15]. Access to medical care, education, and motivation for IIT is influenced by socioeconomic status. This results in a disproportionate percentage of conventional insulin therapy in lower socioeconomic groups.

In practice, only 20–30% of type 1 diabetics are on IIT [16], approximately 10% on an insulin pump and 20% on multiple injections. Therefore, in clinical practice, therapy associated with higher mortality, i.e. conventional insulin therapy, remains the ongoing standard of care. Finally, the requirement for insulin injections, whether conventional or IIT, often has negative psychological and social implications for the patient [17].

## 4. Autologous HSCT: induction of tolerance

### *4.1. Rationale*

The rationale for autologous HSCT is to give a short pulse of intense immune ablative therapy (usually over 4–7 days) followed by infusion of HSC to minimize the duration of myelosuppression (usually 7–11 days following HSC). HSCs are progenitor cells for all immune cells including T and B lymphocytes, macrophages, dendritic cells, neutrophils, and NK cells. In numerous animal models, HSCs depending upon conditions may cause, prevent or cure autoimmune diseases. In general, spontaneous onset animal autoimmune diseases require an allogeneic HSCT to be cured. Environmentally induced animal autoimmune diseases may be cured by a syngeneic or pseudoautologous HSC.

In the early 1980s, lupus prone mice with an intact thymus underwent allogeneic HSC trans-

plantation from normal donors resulting in amelioration or cure of the lupus-like manifestations [18]. Similarly, transplant of HSCs from diabetes prone mice into a normal strain caused diabetes while diabetes in NOD mice may be prevented by allogeneic HSC transplant from a non-disease prone strain [19]. This model system demonstrated that allogeneic HSC depending on source may cause or prevent autoimmune disease. While feasible with animals, allogeneic HSC transplantation was considered to morbid a therapy for patients with chronic autoimmune diseases.

In the 1990s, it was demonstrated that environmentally induced animal autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and adjuvant arthritis (AA) could be cured by syngeneic, autologous or pseudo-autologous (using syngeneic animals in the same stage of disease as the recipient) HSC transplants [20–23]. Since autologous HSC transplants, in contrast to allogeneic HSC transplants, are relatively safe, these animal trials provided a rationale to treat human autoimmune diseases with autologous HSC. Although not yet used to treat diabetes, phase I (safety) autologous HSC transplant clinical trials began 5 years ago for several autoimmune diseases [24]. Phase III (efficacy) trials are now being designed for some human autoimmune diseases [24].

For diabetes, the majority of identical twins are discordant for disease, although the number of diabetes susceptible genes shared between twins may correlate with concordance [25]. This suggests an important causative role for environmental exposure [26] and indicates that autologous HSC transplantation, similar to induced animal autoimmune diseases, may reintroduce tolerance to islet cells in at least a subset of type 1 diabetics.

### 4.2. Risk/benefit

The risk of toxicity from HSC transplant varies by patient selection, conditioning regimen, stem cell source, and supportive care. The regimen of cyclophosphamide (200 mg/kg) and rabbit antithymocyte globulin (ATG) (6.0 mg/kg) with autologous HSC has virtually no mucositis or organ toxicity. The major risk arises from 7–11 days of neutropenia which when treated by pre-emptive antibiotics should result in few serious infectious. At our institution, a cyclophosphamide/ATG regimen has been used safely in 15 patients with systemic lupus erythematosus, all of whom have had astonishing improvements. The anticipated mortality for this regimen in otherwise healthy new onset diabetes could be anticipated to be 1% or less. The only anticipated long term toxicity is age dependent sterility. This may be avoid in males by sperm bank. From experience using the same regimen in aplastic anemia, normal ovarian function and fertility return in all females under 26 years of age but in only 33% over 26 years old [27].

Table 2
Possible criteria for hematopoietic stem cell transplantation of diabetes

| | |
|---|---|
| 1 | Type 1 diabetes |
| 2 | Within 3 months of onset |
| 3 | C-peptide within normal range |
| 4 | No other co-morbid diseases |
| 5 | Ability to sperm bank and understand risk of infertility |
| 6 | Certified by a 3rd party physician to be ineligible or unlikely to be compliant with intensive insulin therapy |

### 4.3. Eligibility

Candidates for an initial protocol of autologous HSC transplant should have new onset type 1 diabetes with detectable C-peptide and determined by a third party physician to be ineligible or unlikely to be compliant with IIT (Table 2). Besides duration of insulin independence, precursor frequency of GAD cytotoxic T cells, and titer of ICA may be followed. If immunologic parameters such as ICA titer normalize but the patient still becomes insulin dependent, the patient may be tolerant to islet cells but have insufficient surviving islet cell mass. In this scenario, islet cell neogenesis may be attempted by a variety of methods (Fig. 1).

## 5. Islet cell regeneration

Stem cell sources for islet cell neogenesis are shown in Fig. 1. Generation from autologous

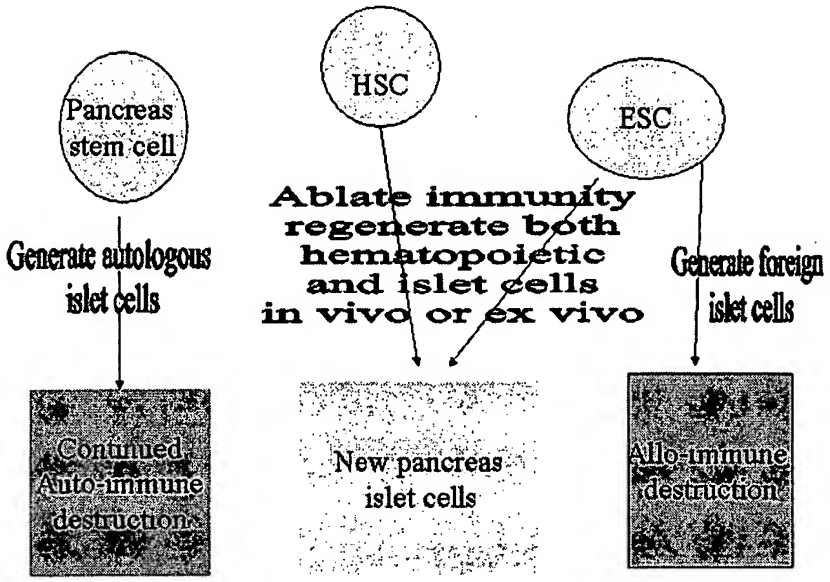


Fig. 1. Stem cell sources for islet cell neogenesis. HSC, hematopoietic stem cell; ESC, embryonic stem cell.

mesenchymal pancreatic stem cells will result in continued autoimmune destruction unless tolerance is induced first. If HSC transplantation can reintroduce islet cell tolerance, islet cell growth factors such as islet neogenesis associated protein (INGAP) may help re-establish normogylcemia without exogenous insulin [28].

Embryonic stem (ES) cells may be used to regenerate both marrow and islet cells. The ES cell derived immune cells should then be tolerant to the genetically identical ES cell derived islet cells. Alternatively, adult HSC may conceivably be a source of both marrow and islet cells. In murine models, it now appears that HSC can change lineage. Under appropriate conditions, murine HSC can be converted into neurons, cardiac myocytes, or hepatocytes [29–32]. It remains unclear if human HSC have the plasticity to switch tissue lineage commitment. Recently, human livers from female recipients of male bone marrow transplant donors were analyzed and Y chromosome specific DNA was detected in a small percentage of hepatocytes [33]. This implies that the male hepatocytes originated from the donor HSC.

The ability of HSC to induce tolerance and possibly repair damaged tissue has practical significance because hundreds of millions of HSC can be easily and repeatedly collected from the peripheral blood by apheresis with little risk to the donor. Autologous HSC are not encumbered by the ethical and immunologic issues associated with embryonic stem cells. Stem cell therapy and specifically HSC therapy to reintroduce tolerance and conceivably repair damaged tissue may become an important new weapon in the therapeutic armamentarium against diabetes.

## Take-home messages

- Type 1 diabetes is an autoimmune disease.
- Cure will require induction of tolerance to islet cells and depending on timing of intervention possibly islet cell regeneration.
- Either adult marrow stem cells or embryonic stem cells may be capable of inducing both tolerance and islet cell regeneration through differentiation into both hematopoietic and islet cells.

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***The World of Autoimmunity; Literature Synopsis***

**Graft-versus-host disease, interferon-gamma and autoimmunity**

Graft-versus-host disease (GVHD) is a known complication of bone-marrow transplantation. Whereas acute GVHD develops in B6D2F1 hybrid recipients of wild-type C57BL/6 parental strain grafts, use interferon-gamma gene knockout donors results in prolongation of the disease in the recipients and a higher level of engraftment, particularly of T cells. Ellison et al. (Immunology 2002;105:63) studied the nature of the latter kind of GVHD characterized by lesions containing large, mixed cellular infiltrates in the skin, liver, pancreas, salivary gland, lung and kidney. They found that spleen cells from these recipients produce IL-4, IL-5 and IL-13 in culture, but respond poorly to concavalin A and lipopolysaccharide. The recipients' sera contain ANA, with specificity in some for dsDNA. There were also eosinophilic infiltrates developing within the target organs. This developed syndrome in the absence of interferon-gamma resembles in certain aspects both chronic GVHD and SLE. It is possible that the absence of interferon-gamma favors the development of an autoimmune-like syndrome in the mice. Of note that chronic GVHD in human subjects also have many features resembling some autoimmune diseases such as systemic sclerosis and Sjogren's syndrome.

**Autoantibodies in Porphyria Cutanea Tarda**

Porphyria cutanea tarda (PCT) is characterized by a deficiency of the cytosolic enzyme uroporphyrinogen decarboxylase which is involved in the synthesis of haem. Whereas this enzyme is deficient in all tissues in familial PCT, its deficiency is confined only to the liver in sporadic PCT. As sporadic PCT is frequently associated with hepatitis C virus (HCV) infection, and HCV infection is associated with autoimmune manifestations, Ma et al. (Clin Exp Immunol 2001;126;47) investigated whether autoimmune reactions are also involved in the pathogenesis of PCT. The authors compared autoantibodies to human cytosolic and microsomal liver fractions between patients with PCT, patients with other liver disorders and healthy subjects. Anti-cytosolic antibodies were more frequent in PCT patients (46%) than in the other groups, and within the PCT group they were more frequent in HCV-positive than-negative patients (57% versus 11%, respectively). Moreover, reactivity towards a 40-kDa cytosolic polypeptide was present in 20 PCT patients, of whom 19 were HCV-positive, more frequent than in any other group. The severity of liver damage and anti-HCV antibodies were also associated with anti-cytosolic antibodies. In contrast, no difference was found in the frequency of anti-liver microsomal antibodies between groups. These results provide another evidence for the contribution of infectious agents in general (and HCV in particular) to autoimmunity, and suggest that sporadic PCT might have an autoimmune component.